REGULATION OF CYTOKINE-INDUCED ADHESION MOLECULE EXPRESSION AND SICKLE ERYTHROCYTE ADHESION TO MICROVASCULAR ENDOTHELIAL CELLS BY INTRACELLULAR ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE AND NITRIC OXIDE

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DEDICATION

For Ben, Mom, and Daddy

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AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
b	Flow chamber width
BCAM	Basal cell adhesion molecule
BSA	Bovine serum albumin
Bt_2cAMP	Dibutyryl cyclic AMP
C	Catalytic subunit
Ca^{2+}	Calcium ion
cAMP	Adenosine 3’,5’-cyclic monophosphate or cyclic AMP
cGMP	Guanidine 3’,5’-cyclic monophosphate or cyclic GMP
Cl^-	Chloride ion
Cont	Continous treatment
DETA-NO	2,2'-(Hydroxynitrosohydrazono)bis-ethanimine
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco’s phosphate buffered saline
ECGF	Endothelial cell growth factor
EDTA	Ethylene diamine tetraacetic acid

EGF	Epithelial Growth Factor
EGM-MV	Endothelial Growth Medium--Microvascular
ELISA	Enzyme linked immunosorbant assay
FBS	Fetal bovine serum
FN	Fibronectin
Fsk	Forskolin
GPIb	Glycoprotein Ib
GVHD	Graft versus host disease
h	Flow chamber gap height
HbF	Fetal hemoglobin
HbSA	Heterozygous sickle hemoglobin (sickle cell trait)
HbSS	Homozygous sickle hemoglobin
Hbßthal	Sickle beta thalassemia
HLA	Human leukocyte antigen
HU	Hydroxyurea
HUVECs	Human umbilical vein endothelial cells
IAP	Integrin associated protein
IBMX	Isobutylmethylxanthine
ICAM-1	Intracellular adhesion molecule 1
ICAM-4	Intracellular adhesion molecule 4
IgGκ	Immunoglobulin
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4

IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
K^+	Potassium ion
L-NMMA	N^G-Monomethyl-L-arginine Acetate salt
MECs	Microvascular endothelial cells
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
O.D.	Optical density
OPD	o-phenylene diamine dihydrochloride
PKA	Protein kinase A
Pre	Pretreatment
PS	Phosphatidylserine
Q	Volumetric flow rate
R	Regulatory subunit
SCA	Sickle cell anemia
SEM	Standard error of the mean
SFM	Serum-free media
SGL	Sulfated glycolipids
SNP	Sodium nitroprusside
SSRBCs	Sickle erythrocytes (red blood cells)
TCD	Transcranial doppler
TNF-α	Tumor necrosis factor alpha
TSP	Thrombospondin

VCAM-1	Vascular cell adhesion molecule 1
vWF	von Willebrand factor
τ	Shear stress
μ	Viscosity

SUMMARY

Adhesion of sickle erythrocytes to vascular endothelium may initiate or propagate occlusive events in sickle cell anemia, many of which are accompanied by infection and the associated inflammatory response. Inflammatory markers are also present in sickle patients during asymptomatic periods. Inflammatory cytokines upregulate expression of endothelial adhesion molecules that promote adhesion of sickle erythrocytes. The data in this work demonstrate that after 2 hrs of stimulation with the cytokine TNF-α, E-selectin, but not VCAM-1 is upregulated on human dermal microvascular endothelial cells. After 6 hrs of TNF-α stimulation, both VCAM-1 and E-selectin expression are upregulated on MECs, and sickle erythrocytes bind to both receptors. Because strategies to control inflammation-associated adhesion *in vivo* may need to account for both VCAM-1 and E-selectin mediated events, control of intracellular signaling pathways leading to receptor expression is an attractive strategy for inhibiting adhesion. Cyclic AMP and nitric oxide are two

intracellular signaling molecules important to cytokine-induced receptor expression. The data in this work demonstrate that TNF-α induced VCAM-1 and E-selectin expression on endothelial cells and sickle erythrocyte adhesion are abated by increasing endothelial cyclic AMP concentrations using Forskolin, IBMX, or Bt_2cAMP. Conversely, when sickle erythrocytes, rather than endothelial cells, are treated with reagents that increase intracellular cAMP, adhesion to unstimulated endothelial cells is increased in some patients. Treatment of endothelial cells with reagents such as SNP and DETA-NO that increase nitric oxide significantly inhibits VCAM-1, but not E-selectin expression, induced by TNF-α stimulation and significantly inhibits sickle erythrocyte adhesion. Treatment of sickle erythrocytes directly with these reagents may also inhibit adhesion. Together these data suggest that cAMP- and nitric oxide-dependent signaling are useful therapeutic targets to inhibit cytokine-induced sickle erythrocyte adhesion to endothelium.

CHAPTER 1

INTRODUCTION

Sickle cell anemia is a genetic disorder estimated to affect 70,000 Americans, primarily of African descent. It is caused by the homozygous inheritance of the sickle β hemoglobin gene, which codes for the amino acid valine in the 6th position rather than glutamic acid. This substitution of a hydrophobic amino acid for a normally hydrophilic one results in the ability of the hemoglobin molecules to aggregate upon deoxygenation. Hemoglobin gelation is the cause of the rigid, “sickle”-shaped erythrocytes for which the condition is named.

Complications associated with sickle cell anemia are systemic, as bones, lungs, brain, spleen, kidneys, heart, eyes, and other organs may be affected in some patients. Vascular complications in sickle cell anemia are multifactorial and complex, affecting both large and small vessels. The most common microvascular problem in tissues is the vaso-occlusive pain episode caused by blood flow

blockage leading to tissue ischemia and severe pain. The abnormal adhesion of sickle erythrocytes to vascular endothelium may initiate or propagate vaso-occlusion. This adherence may slow down blood flow, allowing subsequent entrapment of non-deformable, sickled cells and propagation of the pain episode.

At a post-capillary venular shear stress of 1 dyne/cm^2, sickle erythrocyte adherence to endothelium occurs via binding of specific receptors and ligands on erythrocytes and endothelial cells. Inflammatory mediators (cytokines, chemokines) and thrombotic factors (vWF, etc.) contribute to the development of these high affinity receptor/ligand interactions *in vitro* by increasing cell adhesion molecule expression, receptor affinity, and adhesive protein concentration in the microcirculation. The presence of cytokines and activated monocytes, neutrophils, and platelets in sickle blood during asyptomatic periods and pain events provides *in vivo* support for the roles of inflammation and thrombosis in the pathogenesis of pain episodes.

1.1 Thesis Rationale

The cytokine tumor necrosis factor (TNF-α) induces expression of endothelial receptors VCAM-1, E-selectin, and ICAM-1. VCAM-1 and E-selectin bind specifically to ligands on sickle erythrocytes (ICAM-1 does not), suggesting that strategies to inhibit interactions between sickle erythrocytes and VCAM-1 and E-selectin may be of potential benefit to sickle patients. ***It is hypothesized that sickle erythrocyte adherence induced by inflammatory mediators such as TNF-α is abated by interruption of endothelial cell signaling cascades normally associated with cytokine stimulation.*** Specifically, the aims outlined below probe the roles of secondary messengers cyclic 3′5′-adenosine monophosphate (cAMP) and nitric oxide (NO) in interruption of signaling normally leading to expression of adhesion molecules VCAM-1 and E-selectin in microvascular endothelial cells. These studies suggest therapeutic strategies to reduce erythrocyte adherence and control vaso-occlusion and pain episodes in sickle patients.

1.1.1 Specific Aim #1: Regulate Endothelial Cell Adhesion Molecule Expression and Sickle Erythrocyte Adhesion by Modulation of Intracellular cAMP

Literature suggests that increasing intracellular cAMP in endothelial cells decreases TNF-α induced VCAM-1 and E-selectin expression. Studies in this aim test the effect of forskolin, an activator of the adenylate cyclase enzyme that makes cAMP, isobutylmethylxanthine, an inhibitor of the phosphodiesterases that break down cAMP, and dibutyryl cAMP, a cell-permeable cAMP analogue, on TNF-α induced expression of these adhesion molecules and on sickle erythrocyte adhesion to microvascular endothelium. Specifically, the time course and dose dependent effects of forskolin on endothelial cAMP concentration and adhesion molecule expression are measured using ELISA, and conditions corresponding to maximal effect on adhesion molecule expression are used to test the effect on sickle erythrocyte adherence in flow assays *in vitro*. Antibody blocking of VCAM-1 and E-selectin confirms the roles of these molecules in TNF-α induced adhesion of sickle erythrocytes. The effect of these reagents on sickle erythrocyte adhesion when the erythrocytes, rather than the endothelial cells, are treated is also documented.

Completion of these studies results in 1) establishment of the time course of the relative contributions of VCAM-1 and E-selectin in the promotion of sickle erythrocyte adhesion to TNF-α stimulated microvascular endothelium, 2) demonstration that increasing endothelial intracellular cAMP is effective at reducing cytokine-induced expression of VCAM-1 and E-selectin and adhesion of sickle erythrocytes, and 3) addition of evidence that targeting of intracellular pathways may be useful therapies in controlling vaso-occlusive complications in sickle patients.

1.1.2 Specific Aim #2: Regulate Adhesion Molecule Expression and Sickle Erythrocyte Adhesion to Endothelial Cells by Modulation of Intracellular Nitric Oxide.

Despite its emerging role as an important mediator of several clinical phenomena relevant to sickle cell anemia, little is known about the specific effects of nitric oxide (NO) on sickle cell adherence to endothelial cells. One important potential effect of NO is its ability to interrupt cytokine-induced expression of adhesion molecules on endothelial cells. One goal of the studies in this aim is to harness the ability of NO to regulate adhesion

molecule expression to reduce adhesion of sickle erythrocytes to endothelial cells. Experiments in this aim characterize the effect of the NO donors sodium nitroprusside and 2,2'-(Hydroxynitrosohydrazono)bis-ethanimine (DETA-NO) on TNF-α induced VCAM-1 and E-selectin expression on microvascular endothelium (ELISA) and demonstrate that increasing endothelial NO is effective at reducing adhesion of sickle erythrocytes (flow assays). These experiments also show the effect of treatment of sickle erythrocytes with NO donating compounds on adhesion.

CHAPTER 2

LITERATURE REVIEW

2.1 Sickle Cell Anemia

2.1.1 Etiology

Sickle cell hemoglobinopathies are caused by the genetic inheritance of hemoglobin of altered structure. Hemoglobin protein is comprised of two α subunits, the structures of which are determined by four α globin genes on the 16th chromosome, and two ß subunits, the structures of which are determined by two ß globin genes on chromosome 11 (S. H. Embury *et al.*, 1994). Sickle hemoglobinopathies, two of the most common of which in the United States are sickle cell anemia (HbSS) and sickle ß thalassemia (Hbßthal), result from altered ß globin structure (Bunn & Forget, 1986).

Sickle cell anemia results from the substitution of valine for glutamic acid in the sixth amino acid of the ß globin chains (Pauling *et al.*, 1949). Homozygous

inheritance of this hemoglobin results in sickle cell anemia and clinical symptoms, while heterozygous inheritance results in sickle cell trait (HbSA) and generally few clinical complications (Stark *et al.*, 1980). Sickle ß thalassemia disorders result from inheritance of one sickle ß gene and one ß thalassemia gene. Other common hemoglobinopathies result from inheritance of one sickle ß globin gene and one other mutated ß globin gene. In total, more than 400 abnormal hemoglobin variants are known to exist (Bunn & Forget, 1986), and clinical symptoms may range from non-existent to quite severe. While many clinical similarities exist between the different hemoglobinopathies, the studies presented in this document involve only patients with homozygous sickle cell anemia (SCA).

2.1.2 Natural and Medical History

Origin of the sickle hemoglobin gene and sickle cell anemia has been traced to three regions in Africa: Benin, Senegal, and the Central African Republic (Pagnier *et al.*, 1984). While the time at which the mutation occurred is not known exactly, it likely happened 2,000 to 3,000 years ago, coinciding with the introduction of iron tools, destruction of forests, and the endemicity of the

Plasmodium falciparum malarial parasite (Livingstone, 1958). The inheritance of sickle trait is thought to protect individuals against malarial infections, thus allowing the gene to be passed to future generations (Allison, 1954).

While some evidence points to the recognition by indigenous people of familial pain patterns (Konotey-Ahulu, 1974), little was known about causes of pain or sickle cell anemia until the 20th century. Sickled erythrocytes were first documented in 1910 by Dr. J.B. Herrick, a physician studying the abnormal blood smear of a dental student from Grenada who later experienced a painful crisis (Herrick, mp;list_uids=8919662 </url></related-

he abnormal hemoglobin protein structure of sickle cell anemia by electrophoresis in 1949 (Pauling et al., 1949), but the exact amino acid substitution was not known until 1956 (Ingram, 1956). Studies explaining how altered hemoglobin structure is linked to erythrocyte sickling and clinical symptoms are discussed below.

2.2 Clinical Symptoms

2.2.1 Hemoglobin Aggregation

Unlike normal hemoglobin, sickle hemoglobin has a net positive charge which results in reduced solubility (Perutz & Mitchison, 1950). Upon deoxygenation in the microcirculation, sickle hemoglobin molecules reversibly aggregate into paracrystalline polymers (Magdoff-Fairchild *et al.*, 1976; Noguchi & Schechter, 1981). These aggregates can form long fiber bundles (Dykes *et al.*, 1978), which distort the erythrocyte into the characteristic “sickle” shape described by Herrick (Herrick, 1910).

Aggregation of sickle hemoglobin depends on sickle hemoglobin concentration, oxygen saturation, blood pH, and temperature. Heterozygous sickle cell trait carriers, whose erythrocytes contain a mixture of sickle and non-sickle hemoglobin, exhibit few clinical symptoms and their hemoglobin does not aggregate at physiologic oxygen saturations of 80% (venous circulation) to 95% (arterial circulation) (Ferrone, 1989; Ferrone *et al.*, 1985a, 1985b; Noguchi *et al.*, 1989). However, sickle hemoglobin aggregates are evident at 90% oxygen saturation when hemoglobin concentrations are 42 g/dL, such as are found in dense sickle erythrocytes from homozygous sickle patients

(Noguchi et al., 1989). Hemoglobin aggregation also increases with decreasing pH and increasing temperature (Ferrone et al., 1985a).

Hemoglobin aggregation and erythrocyte sickling upon deoxygenation are not instantaneous. Rather, a delay exists between the time of deoxygenation and the time of hemoglobin aggregation and erythrocyte sickling (Eaton *et al.*, 1976b; Hofrichter *et al.*, 1974). Under normal physiologic circumstances the time required for an erythrocyte to pass through the microcirculation is roughly 1 second (Eaton *et al.*, 1976a), and the delay time is longer, suggesting that most sickle erythrocytes can traverse the microcirculation before becoming stiff and sickled and without occluding microvessels (Mozzarelli *et al.*, 1987). However, factors that either decrease the delay time before onset of hemoglobin aggregation or increase the time for erythrocytes to pass through microvessels could lead to red cell sickling and occlusion in microvessels. Two factors that change the delay time before hemoglobin aggregation are deoxy-sickle hemoglobin concentration and temperature. The delay time is proportional to the negative 30th power of the deoxyhemoglobin concentration. Also, a change of temperature of 1°C in the physiologic temperature range cuts the delay time by nearly half

(Hofrichter et al., 1974). One factor that might result in increased time for erythrocytes to traverse the microcirculation is sludging blood flow due to adhesion of sickle erythrocytes to the vascular endothelium. This is discussed in more detail in Section 2.4.

2.2.2 Hemolytic Anemia

Repeated reversible sickling of erythrocytes due to hemoglobin aggregation increases the membrane rigidity of the erythrocytes due to binding of spectrin to membrane proteins (Lux *et al.*, 1976; Usami *et al.*, 1975). The limited deformability of sickle erythrocytes as they traverse the microcirculation results in mechanical stress and damage to the erythrocytes (Weed, 1975), as evidenced by increased phosphatidylserine presence on the outer leaflet of the erythrocyte membrane (Setty *et al.*, 2002; Yasin *et al.*, 2003). Membrane damage shortens the life-span of sickle erythrocytes from the normal average of 120 days to only 12 to 15 days, resulting in chronic hemolytic anemia (G.R. Serjeant, 1985).

2.2.3 Vaso-Occlusion

Vaso-occlusion occurs when micro or macro vessels are plugged and is the source of many of the clinical symptoms

associated with sickle cell anemia. Vessels may be blocked by several cell types, the most well documented of which include sickle erythrocytes (S. H. Embury *et al.*, 2004; Hebbel, 1997) and leukocytes (Kaul *et al.*, 2004) that abnormally adhere to the endothelial cells lining the vascular wall and stiff, morphologically sickled erythrocytes that lack the flexibility to traverse the microcirculation without entrapment (Ferrone et al., 1985b). The occlusion of vessels with these cell types leads to tissue ischemia, organ damage, and the most serious clinical complications of sickle cell anemia. These clinical complications include pain crises, stroke, acute chest syndrome, retinopathy, and priapism, each of which is described more fully below (Edwards *et al.*, 2005; Maples & Hagemann, 2004; Rogers, 2005).

Pain episodes are periods of diffuse, reversible pain in the back, chest, abdomen, and extremities, especially in the bones. These episodes can last anywhere from a few hours to several weeks, with duration depending on levels of fetal hemoglobin, overall physical condition, psychological and social factors, and the presence or absence of underlying infection (A. Platt et al., 2002). Although pain crises often manifest without apparent precipitants, several factors are known to contribute to

onset of these episodes. These may include physical states such as pregnancy, environmental conditions such as cold temperature or high altitude, psychological states of stress, anxiety, or depression, and pathological states of infection or hypoxia (O. S. Platt *et al.*, 1991).

One of the clearest precipitants of pain episodes and mortality among sickle patients is bacterial infection (Barrett-Connor, 1971; S. H. Embury et al., 1994; Manci *et al.*, 2003; C. Taylor *et al.*, 2004). While the link between infection, the associated inflammatory response, and sickle pain events is not concretely established, evidence presented in Section 2.3 suggests that inflammation contributes to abnormal adhesion of sickle blood cells to the endothelium, which may in turn lead to vacular occlusion and pain.

It is estimated that 10% to 15% of sickle patients suffer some sort of cerebral vascular event (Koshy *et al.*, 1990; Ohene-Frempong *et al.*, 1998) during their lives. Children and older patients are more likely to suffer from infarctive strokes, whereas young adults are more likely to suffer from hemorrhagic strokes (Ohene-Frempong et al., 1998). Strokes represent the second most common cause of death among children with sickle cell anemia (Leikin *et al.*, 1989), and pediatric sickle cell

patients are more than 300 times more likely than non-sickle patients to suffer a stroke (Ohene-Frempong et al., 1998). The mechanisms of stroke in sickle cell anemia are not well understood or documented in the literature. Major risk factors for childhood stroke include anemia, silent infarcts, incidence of acute chest syndrome (C. Hoppe, 2005), and blood velocities of 200 cm/s or higher in the middle cerebral artery or internal carotid artery as determined by transcranial doppler (TCD) (Adams *et al.*, 1997). Autopsies from sickle patients whose cause of death was determined to be from neurologic complications show infarcts and/or hemorrhages in the cerebral hemispheres, thrombosis at major branches of large vessels such as the Circle of Willis or the internal carotid artery, vascular occlusion, and scars from previous microinfarcts (Koshy et al., 1990). Chronic transfusion therapy to decrease sickle hematocrit counts lessens the incidence of stroke in some patients (Pegelow, 2001; Pegelow *et al.*, 2001).

Acute chest syndrome is characterized by chest pain, fever, coughing, and increased respiration (Siddiqui & Ahmed, 2003). It occurs in up to 43% of sickle patients, and 25% of deaths in sickle cell anemia result from acute chest syndrome (Castro *et al.*, 1994; Vichinsky *et al.*, 2000; Vichinsky *et al.*, 1997). Risk factors include age,

high leukocyte counts, low fetal hemoglobin levels, previous history of pulmonary events, and stroke (Castro et al., 1994; C. Hoppe, 2005; Siddiqui & Ahmed, 2003). Known causes of acute chest syndrome include infection, vaso-occlusion, fat embolism, and thrombosis. However, in a study of 537 sickle cell patients who experienced acute chest events, 45% of events occurred with no known cause (Castro et al., 1994; Walker *et al.*, 1979).

Retinopathy in sickle cell anemia occurs due to arteriolar occlusion and ischemia of the peripheral retinal vasculature (Galinos *et al.*, 1975; Talbot *et al.*, 1982). It is estimated that 10 to 20% of sickle patients experience retinopathy and transient visual loss (Moriarty *et al.*, 1988). Permanent vision loss is rare, with one study reporting permanent loss in only one out of 82 eyes affected with retinopathy (Downes *et al.*, 2005).

Priapism, or painful involuntary erection of the penis, occurs in up to half of men with sickle cell anemia (Chinegwundoh & Anie, 2004). There are two types of priapism, stuttering attacks which occur intermittently and range from several minutes to hours in duration and acute attacks which last from hours to days (Mantadakis *et al.*, 1999; Maples & Hagemann, 2004; Rogers, 2005). Most incidences of priapism in sickle cell anemia are

characterized by low blood flow and decreased outflow from penile veins. This may be due to sickling of erythrocytes within the corpora cavernosa of the penis and subsequent venous stasis (Maples & Hagemann, 2004). However, some cases of priapism in sickle cell patients are characterized by high blood flow as well (Ramos *et al.*, 1995). Priapism lasting for many hours can result in irreversible cell damage and impotence (Rogers, 2005). Treatment options for low-flow priapism are generally pharmacologic in nature, with adrenergic agents representing one major category. High-flow priapism tends to require arterial embolization or ligation (Maples & Hagemann, 2004).

Hemoglobin aggregation, anemia, and vaso-occlusion are the three major physiologic factors affecting sickle cell anemia patients. In general, the anemia is relatively well-tolerated, whereas vaso-occlusion is responsible for most clinical disease manifestations and the morbidity and mortality associated with the disorder (G. R. Serjeant, 1993). The role of the endothelium in the progression of clinical events in sickle cell anemia is discussed in the following section.

2.3 The Endothelium in Sickle Cell Anemia

The vascular endothelium plays an important part in the physiology and progression of clinical events in sickle cell anemia. The endothelium is responsible for regulating vascular tone and providing a barrier between blood and tissue. In addition, the endothelium is a critical mediator of the inflammatory response, expressing receptors for leukocyte recruitment, attachment, and infiltration. Finally, the endothelium produces plasma proteins that are critical to the coagulation cascade. The endothelium in sickle patients is unique in that it is abnormally activated to express receptors and proteins involved in inflammation and coagulation and has diminished capacity to regulate vascular tone (Hebbel *et al.*, 2004). These aspects of sickle biology are discussed in more detail below.

2.3.1 Vasoregulation

The endothelium regulates vascular tone by producing vaso-active substances including dilators such as nitric oxide (NO) and prostaglandins as well as contrictors such as endothelin-1. The sickle circulation is characterized by hypoperfusion leading to tissue ischemia in the

microcirculation (Kaul *et al.*, 1996; Kaul *et al.*, 2000; Nath *et al.*, 2004; Osarogiagbon *et al.*, 2000), but hyperperfusion in the macrocirculation (Gladwin *et al.*, 2003; Hatch *et al.*, 1989; Nath et al., 2004), suggesting overall vascular instability and inability to regulate vascular tone.

While mechanisms for this vascular instability are not well-understood, it appears that one major contributing factor is decreased bioavailability of nitric oxide (Morris *et al.*, 2000a; Morris *et al.*, 2000b). Nitric oxide is produced by nitric oxide synthases (NOS) in endothelial cells, then diffuses to smooth muscle cells where it initiates cell signaling eventually resulting in calcium sequestration and vessel relaxation (Nath et al., 2004). The bioavailability of and responsiveness to nitric oxide appears greater in women with sickle cell anemia than men with the condition (Gladwin et al., 2003). Nitric oxide deficiency is a multifaceted problem that may be caused by several factors loosely grouped as oxidative damage but more specifically including the following:

- Consumption of nitric oxide by the elevated amounts of hemoglobin in sickle plasma (Reiter *et al.*, 2002)

- Consumption of nitric oxide from excessive superoxide generation (Osarogiagbon et al., 2000)
- Uncoupling of endothelial NOS by oxidation of tetrahydrobiopterin (Landmesser *et al.*, 2003)
- Activation of xanthine oxidase (Aslan *et al.*, 2001; Nath et al., 2004)

In addition to serving as a vasoregulator, nitric oxide may significantly affect other biological phenomena important to sickle cell anemia, such as receptor expression and cell adhesion (De Caterina *et al.*, 1995; Gladwin & Schechter, 2001). These are discussed in more detail in Section 2.7 below.

2.3.2 Coagulation

Unlike quiescent endothelium of non-sickle patients, which is generally nonthrombogenic, endothelium of sickle patients shows signs of chronic hypercoagulability that is further exacerbated during clinical events (S. H. Embury et al., 1994). Sickle cell anemia patients exhibit increased rates of thrombin and fibrin formation (Francis, 1989; Leslie *et al.*, 1975) and consumption of anti-thrombogenic substances such as antithrombin 2I, protein C, and protein S (Cacciola *et al.*, 1989; Francis, 1988; Green

& Scott, 1986; Karayalcin & Lanzkowsky, 1989). Tissue factor, the trigger for onset of coagulation, is elevated on circulating endothelial cells of sickle patients (Key *et al.*, 1998; A. Solovey *et al.*, 1998), and platelet counts are slightly elevated in sickle patients (Westwick *et al.*, 1983).

Hypercoagulation promotes platelet adhesion, aggregation, and thrombus formation and may contribute to vaso-occlusive events in sickle cell patients. While many aspects of clinical events in sickle cell anemia are not well understood, some studies suggest a link between thrombosis and occurrence of stroke in sickle patients (Prengler *et al.*, 2002).

2.3.3 Inflammation

Sickle cell anemia patients experience chronic inflammation that may be exacerbated further during clinical events. The causes of inflammation remain unclear, but likely include the following:

- *Infection*. Clinical events in sickle cell anemia are often preceded or accompanied by infection (S. H. Embury et al., 1994; Manci *et al.*, 2003; C. Taylor *et al.*, 2004). Infections induce

inflammatory signaling cascades, leading to the signs of inflammation noted below.

- *Adhesion of Sickle Erythrocytes to Vascular Endothelium*. Sickle erythrocytes can induce endothelial activation and expression of adhesion molecules normally associated with inflammation, such as VCAM-1, E-selectin, and ICAM-1 *in vitro* (M. D. Brown *et al.*, 2001; Shiu *et al.*, 2000).
- *Clinical events resulting in tissue ischemia and damage*. While inflammation likely contributes to the onset of occlusion and clinical events, it is also likely that occlusive events exacerbate inflammation, creating a vicious cycle for sickle patients (Hebbel et al., 2004). The process of reperfusion during resolution of occlusion damages the endothelium and triggers inflammatory signaling characteristic of ischemia/reperfusion injuries. Transgenic sickle mice provide *in vivo* evidence that hypoxia/reoxygenation induces inflammation, as they display increased adhesion of leukocytes to vascular endothelial cells (Kaul & Hebbel, 2000).

Much evidence exists to suggest that the sickle endothelium exists in a persistently activated state.

Sickle subjects display elevated levels of inflammatory cytokines such as TNF-α and various interleukins (IL1ß, IL4, IL6, and IL10) (Hibbert *et al.*, 2005; Malave *et al.*, 1993; Pathare *et al.*, 2004; S. C. Taylor *et al.*, 1995; S. C. Taylor *et al.*, 1997). Circulating endothelial cells from sickle patients show presence of inflammatory response receptors including VCAM-1, E-selectin, ICAM-1, and P-selectin (A. Solovey *et al.*, 1997). Soluble VCAM-1, E-selectin, and ICAM-1 levels are also elevated (Conran *et al.*, 2004; Duits *et al.*, 1996; Mohan *et al.*, 2005b; Sakhalkar *et al.*, 2004). Other signs of inflammation include increased leukocyte (Awogu, 2000; Okpala, 2004) and platelet counts (Mohan *et al.*, 2005a) and activated monocytes (Belcher *et al.*, 2000; Lard *et al.*, 1999; Wun *et al.*, 2002).

Transgenic sickle mice also appear to exhibit an inflammatory phenotype, expressing increased amounts of IL-6, VCAM-1, ICAM-1, E-selectin, P-selectin, and tissue factor (Belcher *et al.*, 2003; S. H. Embury et al., 2004; A. Solovey *et al.*, 2004; K. Wood *et al.*, 2004a; K. C. Wood *et al.*, 2004b). In addition, sickle mice demonstrate increased adhesion of sickle erythrocytes to endothelium (S. H. Embury et al., 2004) and rolling and adhesion of leukocytes than non-sickle controls (K. C. Wood et al.,

2004b). The anti-inflammatory drug sulfasalazine inhibits leukocyte adhesion and improves microvascular flow in transgenic sickle mice (Kaul *et al.*, 2004), suggesting that transgenic sickle mice are useful for testing therapeutics (Hebbel et al., 2004) and that anti-inflammatory drugs may benefit sickle patients.

2.4 Sickle Erythrocytes

2.4.1 Sickle Erythrocyte Abnormalities

As established in Sections 2.2 and 2.3, both sickle erythrocytes and endothelial cells from sickle patients are abnormal. The erythrocyte population in sickle patients is quite heterogeneous. Some erythrocytes are irreversibly sickled and do not assume the normal biconcave shape upon reoxygenation. Irreversible sickling is attributed to spectrin-actin cytoskeletal rearrangement and increased binding of sickle hemoglobin to the erythrocyte membrane (Evans & Mohandas, 1987; Lux et al., 1976). Other erythrocytes containing sickle hemoglobin reversibly become stiff and sickled upon deoxygenation (Magdoff-Fairchild et al., 1976; Noguchi et al., 1989; Noguchi & Schechter, 1981) and may occlude microvessels if the delay time between hemoglobin aggregation and erythrocyte sickling is shorter

than the transit time through the microcirculation (Eaton *et al.*, 1976a; Mozzarelli et al., 1987). In addition, the membranes of some erythrocytes are damaged by repeated sickling, altering the lipid organization of the membranes and resulting in exposure of phosphatidylserine (PS) on the outer leaflet (Yasin et al., 2003). This abnormal exposure of PS creates a negatively charged and pro-thrombogenic cell surface (Zwaal *et al.*, 1989). Other membrane glycoproteins such as band 3 and glycophorin A were found to be aggregated on the surface of some sickle erythrocytes (Corbett & Golan, 1993; Liu *et al.*, 1996; Thevenin *et al.*, 1997).

One other important group of erythrocytes in sickle blood is reticulocytes, or immature erythrocytes recently released from the bone marrow. By age 5, sickle patients average a reticulocyte count of 12% (A. K. Brown *et al.*, 1994; el Sayed & Tawfik, 1994), whereas non-sickle subjects average a reticulocyte count of about 1%. The increase is likely an attempt to compensate for the shortened erythrocyte lifespan and overall reduced erythrocyte counts and anemia experienced by sickle patients. Maturation of reticulocytes occurs after release from bone marrow, and until this happens, the membranes of these cells contain transferrin (de Jong *et al.*, 2001) and fibronectin (Patel

et al., 1985) receptors that older cells do not. In addition, sickle reticulocytes have been shown to express $\alpha_4\beta_1$ (Swerlick *et al.*, 1993) as well as CD36 (Joneckis *et al.*, 1993).

2.4.2 Mechanisms of Sickle Erythrocyte Adhesion to Endothelium

Sickle erythrocytes are abnormally adherent to vascular endothelium. Adhesion has been demonstrated *in vivo* in sickle transgenic mice as well as in numerous *in vitro* studies elucidating specific adhesion mechanisms (S. H. Embury et al., 2004; Hebbel *et al.*, 1980; Hoover *et al.*, 1979; Kaul *et al.*, 1995). Erythrocyte adhesion is likely important to the clinical course of sickle cell anemia in that it is thought to increase the transit time of erythrocytes through the microcirculation, allowing sickling of erythrocytes in the microcirculation and initiating vaso-occlusion (Eaton et al., 1976a; Hofrichter et al., 1974). Sickle erythrocytes may be more adherent to the endothelium of sickle patients because the endothelium may be abnormally activated to express higher levels of adhesion molecules as discussed in Section 2.3 and because the sickle erythrocytes abnormally display ligands as discussed in Section 2.4.1 that bind to these receptors.

Specific receptor/ligand interactions supporting adhesion of sickle erythrocytes have been identified using *in vitro* assays where variables such as flow conditions and cell activation states are easily controlled. A summary of these interactions is provided below.

2.4.2.1 Direct Adhesion of Sickle Erythrocytes to Endothelial Cells. The adhesion of sickle erythrocytes to vascular endothelium may occur by direct interaction of erythrocyte ligands with endothelial adhesion molecules or through interactions of sickle erythrocyte ligands and endothelial adhesion molecules with bridging molecules. Mechanisms of binding of sickle erythrocytes directly to endothelial cells (without bridging molecules) are listed below.

- The best-characterized example of direct adhesion of sickle erythrocytes to endothelial cells occurs via binding of a_4b_1 on sickle erythrocytes to endothelial **VCAM-1**. VCAM-1 expression on endothelial cells is stimulated by inflammatory cytokines such as TNF-α (Swerlick *et al.*, 1993).
- When a_4b_1 on sickle erythrocytes is activated by phorbol ester or IL8, it binds to **surface associated**

fibronectin (FN) on endothelial cells (Kumar *et al.*, 1996a).

- Externalized **phosphatidylserine** on sickle erythrocytes binds to microvascular endothelial **CD36** (Closse *et al.*, 1999; Setty et al., 2002). CD36 is not expressed on large vessel endothelium (H. A. Brittain *et al.*, 1992).
- **Aggregated band 3** on sickle erythrocytes binds to **unknown receptor(s)** on endothelial cells (Thevenin *et al.*, 1997).
- **Unknown ligands** on sickle erythrocytes bind to endothelial **P-selectin and E-selectin**. P-selectin is upregulated on endothelial cells by stimulation with histamine or thrombin, and E-selectin is upregulated by stimulation with inflammatory cytokines such as TNF-α (Matsui *et al.*, 2001; Natarajan *et al.*, 1996).
- Stimulation of sickle erythrocytes with epinephrine activates **ICAM-4**, which binds to endothelial $a_V b_3$ (Zennadi *et al.*, 2004).

2.4.2.2 Adhesion of Sickle Erythrocytes to Endothelial Cells via Bridging Molecules. In addition to binding directly to endothelial cells, sickle erythrocytes may also

bind via a mediator, or bridging molecule. Thrombospondin and unusually large molecular weight von Willebrand factor are two plasma proteins serving important mediating roles in sickle erythrocyte/endothelial interactions. The contribution of each to sickle cell adhesion is discussed in this section.

- **Thrombospondin (TSP)** is a large glycoprotein secreted by platelets (Lawler *et al.*, 1978) and endothelial cells (Kramer *et al.*, 1985) that interacts with cell surface receptors on both endothelial cells and sickle erythrocytes via its multiple binding sites (Gupta *et al.*, 1999). TSP receptors on sickle erythrocytes include **CD36** (Sugihara *et al.*, 1992), **sulfated glycolipids** (SGL) (Hillery *et al.*, 1996), **phosphatidylserine** (Setty et al., 2002), and **integrin associated protein** (IAP) (J. E. Brittain *et al.*, 2001a, 2001b). Endothelial receptors include **CD36** (H. A. Brittain *et al.*, 1993; Sugihara et al., 1992), $a_V b_3$ (H. A. Brittain et al., 1993; Kumar *et al.*, 1996b), and **sulfated heparin** (Gupta et al., 1999).
- **Unusually large molecular weight von Willebrand factor (vWF)** multimers bridge **GPIb-like** and integrin receptors on sickle red cells and similar receptors on

endothelial cells (Wick *et al.*, 1987; Wick *et al.*, 1993).

2.4.2.3. Adhesion of Sickle Erythrocytes to Sub-endothelial Matrix Proteins. In addition to adhering to endothelial cells, sickle erythrocytes can also bind to endothelial matrix proteins. This binding may be relevant *in vivo*, since the endothelium is thought to be damaged in sickle patients, and, thus, the underlying matrix may be exposed (Hines *et al.*, 2003; Kasschau *et al.*, 1996; A. Solovey et al., 1997). Mechanisms of binding of erythrocytes to matrix proteins are listed below.

- Sickle erythrocytes adhere to immobilized **FN** via a_4b_1 (Kasschau *et al.*, 1996).
- Sickle erythrocytes adhere to immobilized **laminin** via **BCAM/Lutheran protein** that is activated by epinephrine stimulation (Hines *et al.*, 2003).

Sickle erythrocytes are abnormally adherent to vascular endothelium. Adhesion occurs via direct contact of endothelial adhesion molecules with ligands on sickle erythrocytes or via contact of endothelial adhesion molecules and sickle erythrocyte ligands with bridging proteins. Sickle erythrocytes are also abnormally adherent

to endothelial matrix proteins. A summary of the molecular interactions between sickle erythrocytes and endothelial cells and matrix proteins is given in Figure 2.1 below.

The mechanisms of adhesion outlined above have been identified under a variety of flow conditions. A decription of the flow environment of the sickle microvasculature as well and how this may affect sickle cell adhesion is given in Section 2.4.3 below.

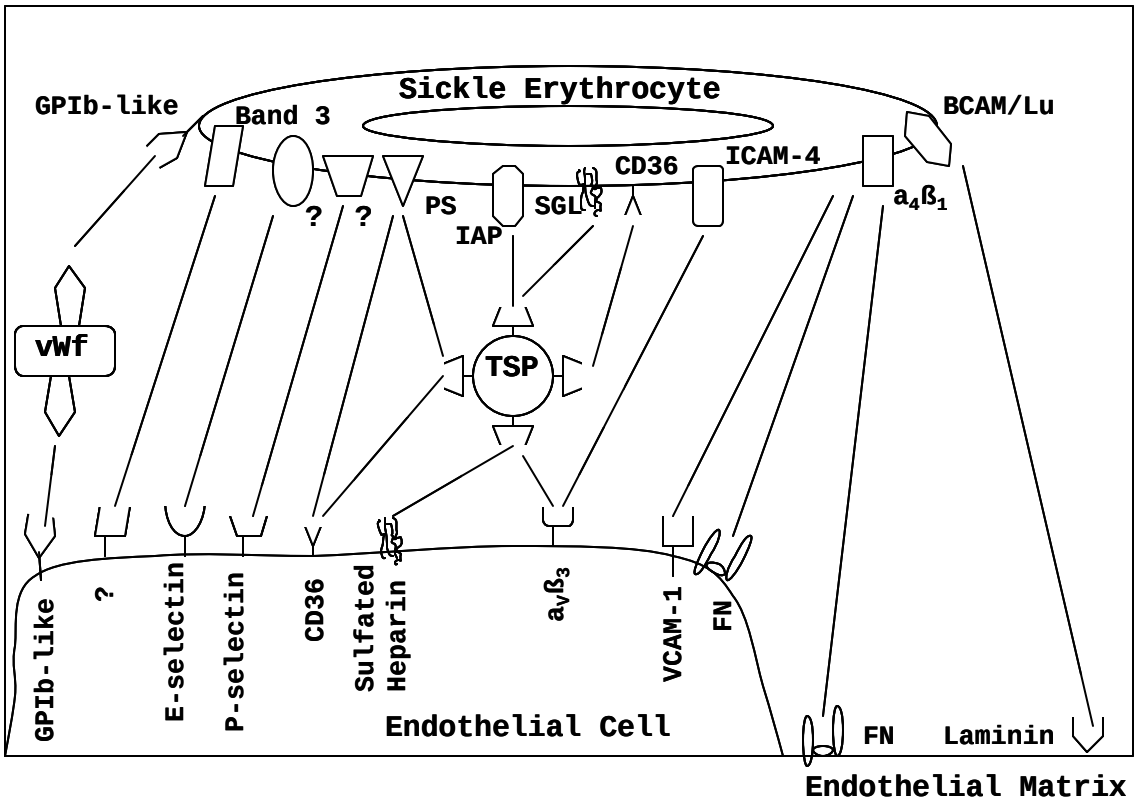


Figure 2.1 Mechanisms of adhesion between sickle erythrocytes and endothelial cells and matrix. Sickle erythrocytes adhere to endothelial cells directly or via bridging molecules. They also bind to endothelial matrix proteins.

2.4.3 Hemodynamics of Sickle Erythrocyte Adhesion

Sickle erythrocyte adhesion is thought to occur primarily in the post-capillary venules (Kaul *et al.*, 1989). The shear stress in these venules for non-sickle patients is estimated to be 1 dyne/cm^2 (Turitto, 1982). Many *in vitro* assays have shown minimal adhesion of non-sickle erythrocytes at this shear stress, but increased adhesion of sickle erythrocytes (Barabino *et al.*, 1987; Wick et al., 1987). These assays have proven quite useful at identifying specific adhesion molecule/ligand interactions that support sickle erythrocyte adhesion. However, some studies suggest that this flow condition may not be the prevalent one in the sickle microvasculature. Rather, conditions of oscillatory flow and frequent stasis have been demonstrated (Lipowsky *et al.*, 1987; Rodgers *et al.*, 1984).

Differences between hemodynamics in sickle and non-sickle patients are further exemplified by erythrocyte velocity profiles. Sickle patients show broad histograms of red cell velocities through the microcirculation, where non-sickle patients show narrower histograms (Lipowsky et al., 1987), meaning that some sickle erythrocytes move through the microcirculation slowly. These results are mimicked in parallel plate flow chambers *in vitro*, which

demonstrate a population of erythrocytes that “slide” or “roll” along the endothelial surface (Matsui et al., 2001; Matsui *et al.*, 2002). P-selectin induces this sliding effect, but it is not known if other adhesion molecules are also responsible. Also unknown is whether or not the sliding results in increased capture and firm adhesion of sickle erythrocytes, just as rolling of leukocytes aids in their capture (Hebbel et al., 2004).

In order for sickle erythrocytes to firmly adhere to the endothelium, the bonding force must be stronger than the detaching forces of fluid shear stress and peeling torque. The strength of the bonding force depends on both the number of bonds between erythrocyte and endothelial cell molecules as well as the strength of each individual bond (Hebbel, 1997; Hebbel et al., 2004). Under fluid flow at the typical post-capillary venule shear stress of 1 dyne/cm^2, sickle cell adherence requires the presence of specific, high-affinity bonds between endothelial adhesion molecules and sickle erythrocyte ligands such as those outlined in Section 2.4.2. These bonds are strong enough collectively to withstand the opposing shear forces. Thus at venular shear stresses, sickle cell adhesion is said to be “affinity-controlled.” Under conditions of low flow (0.1 dyne/cm^2), however, fluid flow opposing adhesion is low

and, thus, low-affinity bonds, the identities of which are not well-characterized, are sufficient to support significant sickle erythrocyte adhesion. Adhesion under low flow conditions is said to be “transport-controlled,” because the level of adherence depends primarily on the contact time between sickle erythrocytes and endothelial cells (Montes *et al.*, 2002; Wagner *et al.*, 2004).

Whether high-affinity or low-affinity, sickle erythrocyte adhesion to endothelial cells is important as a likely initiator of vaso-occlusion in sickle cell anemia. Thus strategies to control or eliminate abnormal adhesion represent an attractive therapeutic prospect for sickle cell anemia patients. To date, no such therapies exist. The following section outlines current cures and therapies available for sickle cell patients and also addresses potential future therapies, including strategies to prevent sickle erythrocyte adhesion.

2.5 Current and Future Therapies

Despite the significant collective body of knowledge regarding sickle cell syndromes, treatment options are still quite limited, owing to the complex pathology of the

condition. For most patients, the primary treatments are merely preventative and supportive. Treatment of acute pain episodes generally includes control of pain with opiates such as morphine, intracellular hydration with oral or intravenous fluids, correction of hypoxia and acidosis, bed rest, and treatment of underlying infection or other complications (A. Platt *et al.*, 2002). However, for those with severe complications, three major therapeutic options are currently available.

2.5.1 Bone Marrow Transplant

Bone marrow transplant is currently the only available curative therapy for sickle cell anemia. While the survival rate from this procedure is roughly 91% and the cure rate is 82% (C. C. Hoppe & Walters, 2001), the option of bone marrow transplantation is currently limited primarily to children under 16 years of age with severe, pre-existing complications. One major barrier to transplants is the lack of a suitable donor for the vast majority of patients; it is estimated that only 14% of patients have a human leukocyte antigen (HLA)-matched sibling donor (C. C. Hoppe & Walters, 2001). Another obstacle is the potential for short and long-term complications after transplantation including intracerebral

hemorrhage, graft-versus-host disease (GVHD), seizure, and gonadal dysfunction (Amrolia *et al.*, 2003; C. C. Hoppe & Walters, 2001).

2.5.2 Blood Transfusion

Blood transfusion is widely used in the treatment of sickle cell anemia. It is estimated that 50% of all patients receive a red cell transfusion at some point in their lives and that 5% receive chronic transfusions (Rosse *et al.*, 1990). Serious complications associated with this therapy include iron overload, allo-immunization, and risk of transmitted infection (Amrolia et al., 2003).

2.5.3 Hydroxyurea

Hydroxyurea is a therapeutic agent administered to patients with severe sickle cell symptoms. It was originally administered due to its ability to increase fetal hemoglobin (HbF) concentrations, which interferes with sickle hemoglobin (HbS) aggregation and red cell sickling both by preventing effective contact between adjacent HbS molecules and by forming mixed HbF-HbS hybrids which are more soluble than HbS aggregates (Halsey & Roberts, 2003). Additionally, hydroxyurea reduces sickle erythrocyte expression of $\alpha_4\beta_1$ and CD36 (Styles *et al.*,

1997) and *in vitro* adhesion to endothelial thrombospondin and laminin (Hillery *et al.*, 2000). The drug also increases erythrocyte hydration and deformability (Ballas *et al.*, 1989) and decreases neutrophil counts (O. S. Platt *et al.*, 1994). Recently, hydroxyurea metabolism has been shown to lead to nitric oxide (NO) release (J. Jiang *et al.*, 1997), and it is possible that some of the drug's effects may be mediated by this release. Although the effects of hydroxyurea are of broad scope *in vitro*, many of these effects have not yet been linked directly to patient benefit, and it is unclear which effects are most relevant to sickle cell anemia pathology. Common short-term side effects of hydroxyurea usage include dose-dependent myelosuppression, nausea, vomiting, and skin rashes. The most troublesome long-term potential risk may be leukemogenicity (Amrolia et al., 2003).

2.5.4 Gene Therapy

As noted earlier, currently the only cure for sickle cell anemia is bone marrow transplant, which works by ablating the patient's hematopoeitic stem cells and replacing them with those from a donor. Since most sickle cell patients do not have a suitable donor, gene therapy represents an attractive curative therapy. It is

different, in that autologous bone marrow cells are used. The process involves removing bone marrow from the patient, transferring viral vectors containing normal β-globin into the cells, selecting out the cells that are successfully corrected, and then transplanting the corrected cells back into the patient. The biggest challenge for this therapy is developing more efficient levels of gene transfer and increasing the levels of gene expression (Bank *et al.*, 1989). Recently, results from murine studies of β-thalessemia in which a lentivirus vector was used to transfect cells showed such positive results in terms of high gene transfer and expression rates, that a Phase I/II clinical trial is currently under way (Bank *et al.*, 2005).

2.5.5 Anti-sickling Agents

Another potential therapy for sickle cell anemia patients is inhibition of sickle hemoglobin aggregation and erythrocyte sickling. Prevention of sickling may reduce the erythrocyte damage and death that leads to chronic anemia and prevent occlusion of vessels with sickled cells. As noted in Section 2.2.1, hemoglobin aggregation and onset of sickling are strongly dependent on sickle hemoglobin concentration (Hofrichter et al., 1974). Thus two main

strategies are used to decrease sickle hemoglobin concentration and erythrocyte sickling.

One strategy is to increase the concentration of fetal hemoglobin in erythrocytes, since fetal hemoglobin does not form aggregates. Fetal hemoglobin levels are inversely correlated with vaso-occlusive complications in sickle cell patients. It is estimated that a fetal hemoglobin concentration of 10% ameliorates major organ failure, while a concentration of 20% is required to ameliorate vaso-occlusive crisis, pulmonary disorders, and other recurrent symptoms (Powars *et al.*, 1984). As noted in Section 2.3.3, one effect of hydroxyurea is increased fetal hemoglobin production (Halsey & Roberts, 2003). However, the potentially cytotoxic, clastogenic (breaking of chromosomes), carcinogenic, and mutagenic properties of the drug mean that it cannot be administered to all patients (Amrolia et al., 2003; Charache *et al.*, 1987). Another, less cytotoxic drug, Decitibine, has also been shown to increase fetal hemoglobin concentrations from 6.5% to 20.4% and reduce reticulocyte counts from 231 to 163 x 10^9 cells per L in a small sample of patients (Buchanan *et al.*, 2004; Sauntharajah *et al.*, 2003). However, one animal study suggests that the drug may also be carcinogenic (Sauntharajah & DeSimone, 2004). Other fetal hemoglobin

inducing compounds include short chain fatty acids, which inhibit histone deacetylase, causing histone hyperacetylation, changes in chromatin structure, and enhancement of fetal hemoglobin gene expression in sickle cell patients. In Phase II clinical trials, administration of arginine butyrate increased fetal hemoglobin levels from 7% to 21% in 11 of 15 patients studied (Atweh *et al.*, 1999; Buchanan et al., 2004).

The other main strategy for decreasing sickle hemoglobin concentrations in erythrocytes and inhibiting hemoglobin aggregation and erythrocyte sickling is hydration of erythrocytes. Sickle erythrocytes are dehydrated due to loss of potassium (K^+) and chloride (Cl^-) ions as well as water (Glader *et al.*, 1978; Glader & Nathan, 1978). Oral magnesium supplements have been shown to inhibit co-transport of K^+ and Cl^- ions *in vivo*, and a clinical trial demonstrates that magnesium improves erythrocyte hydration and reduces the percentage of days that sickle cell anemia patients experience pain (Buchanan et al., 2004; De Franceschi *et al.*, 2000). The efflux of K^+ ions from erythrocytes also occurs through Ca^{2+}-activated "Gardos" channels (Gardos, 1958). A recent study demonstrated that blocking of these channels by ICA 17043, a more potent and less toxic derivative of the drug

Clotrimazole, resulted in decreased sickle erythrocyte density and hemolysis. Clinical benefit from the treatment was not established (Brugnara, 2003; Buchanan et al., 2004; Stocker *et al.*, 2003).

2.5.6 Anti-coagulation Therapy

As established in Sections 2.3.2 and 2.4, sickle cell anemia patients exhibit activated coagulation systems that may result in increased sickle erythrocyte adhesion to vascular endothelium. Thus some have hypothesized that therapies which inhibit thrombosis or coagulation may benefit sickle cell patients. Early reports indicate that ticlopidine (Cabannes *et al.*, 1984; Lonsdorfer *et al.*, 1984), warfarin (Ahmed *et al.*, 2004; Salvaggio *et al.*, 1963), and minidose heparin (Chaplin *et al.*, 1989) may benefit sickle patients experiencing pain episodes, while low-intensity coumadin (Schnog *et al.*, 2002; Wolters *et al.*, 1995) and aspirin (Greenberg *et al.*, 1983; Zago *et al.*, 1984) do not (Hebbel et al., 2004).

2.5.7 Anti-inflammatory Therapy

Given that in addition to coagulation, sickle patients also experience chronic inflammation that may contribute to increased sickle erythrocyte adhesion and clinical

symptoms, anti-inflammatory therapies may benefit sickle patients. The Hebbel group recently demonstrated that patients taking sulfasalazine, an anti-inflammatory agent that inhibits transcription factor nuclear factor kappa B (NF-κB) that is necessary for transcription of many inflammation-induced proteins (Ghersa *et al.*, 1994), showed decreased expression of adhesion molecules VCAM-1, E-selectin, and ICAM-1 on their circulating endothelial cells (A. A. Solovey *et al.*, 2001). Given this success, the group proposes a clinical trial combining the use of sulfasalazine and statins (Hebbel et al., 2004), which provide anti-inflammatory benefits and improve NO availability in non-sickle models (Koh, 2000). To date, no such trial has been conducted.

The studies presented in the following chapters explore the effects of anti-inflammatory compounds on adhesion molecule expression and adhesion of sickle erythrocytes to vascular endothelium. Cyclic AMP and nitric oxide are two important cell signaling molecules that regulate inflammatory responses in endothelial cells. The roles of these molecules in cell signaling, endothelial biology, and sickle cell anemia are discussed in Sections 2.6 and 2.7 below, and their effect on sickle erythrocyte adhesion is the subject of Chapters 5 and 6.

2.6 The Role of cAMP in Endothelial Biology and Cell Signaling

Cyclic AMP is an important and widespread mediator of the conversion of extracellular signals into intracellular events (Galea & Feinstein, 1999). The mechanism of its action has been well-studied, and many key components of the signaling pathway have been identified. The binding of extracellular ligands to some G-protein-coupled receptors results in release of G-proteins that activate the enzyme adenylate cyclase. Adenylate cyclase converts bound ATP to cAMP (Iyengar, 1993). The cAMP activates protein kinase A (PKA), a tetramer of two catalytic (C) and two regulatory (R) subunits. In the absence of cAMP the R_2C_2 protein is catalytically inactive. Upon binding of cAMP to the R subunits, the R and C subunits dissociate, and the C subunits become catalytically active. The active C subunits of PKA phosphorylate a wide variety of intracellular proteins (Habener). Active C subunits may also enter the nucleus (Nigg *et al.*, 1985), where they phosphorylate and activate transcription factors (Montminy, 1997). Cyclic AMP-mediated signaling is generally transient and followed by a refractory period (Armstrong *et al.*, 1995). Deactivation of cAMP signaling may occur by

desensitization of G-coupled receptors, degradation of cAMP by phosphodiesterases, inhibition of PKA, and/or activation of dephosphorylating phosphatase enzymes (Bushnik & Conti, 1996; Frohman *et al.*, 1988; Iyengar, 1993).

In experiments, activation of cAMP-dependent signaling has traditionally been achieved by several methods including 1) activation of G-coupled receptors, 2) direct activation of adenylate cyclase by forskolin, 3) inhibition of cAMP degradation using phosphodiesterase inhibitors such as rolipram, 4) administration of membrane-permeable cAMP analogs (dibutyryl cAMP and 8-bromo-cAMP) that are resistant to degradation by phosphodiesterases, and 5) inhibition of phosphatases with okadaic acid (Galea & Feinstein, 1999).

Several studies point to the role of cAMP as a ubiquitous regulator of inflammatory and immunological reactions. Increases in cAMP have been shown to block T lymphocyte activation (Birch & Polmar, 1982), inhibit interleukin 1β (IL-1β) and TNF-α release from macrophages (Knudsen *et al.*, 1986; Kunkel *et al.*, 1988), and reduce expression of adhesion molecules in endothelial cells (Pober *et al.*, 1993). Specifically, increasing cAMP reduces expression of VCAM-1 and E-selectin, but not ICAM-1, on TNF-α stimulated endothelial cells (Otsuki *et al.*,

2001; Pober et al., 1993). These and other studies have led to the notion that one physiological role of cAMP-dependent signaling is suppression of immune responses (Galea & Feinstein, 1999).

The exact mechanism by which inhibition of adhesion molecule expression by cAMP occurs is not completely understood. It has been shown that raising cAMP levels inhibits transcription of VCAM-1 and E-selectin mRNA (Ollivier *et al.*, 1996; Otsuki et al., 2001). Literature suggests that nuclear factor κB (NF-κB) is a transcription factor important to both VCAM-1 and E-selectin expression. NF-κB is normally stored in an inactivated state (that is, bound to the inhibitory protein IκBα) outside the cell nucleus, but is dissociated from IκBα and is translocated to the nucleus upon stimulation with cytokines such as TNF-α (Ollivier et al., 1996). One study suggests that raising cAMP levels does not prevent nuclear translocation of NF-κB, but it does prevent NF-κB from binding to the VCAM-1 promoter region (Ollivier et al., 1996; Otsuki et al., 2001). Figure 2.2 shows a proposed pathway for VCAM-1 and E-selectin gene transcription and how cAMP interrupts this pathway.

Given the ubiquitous nature of cAMP, it is not surprising that it affects expression of other receptors and ligands important to sickle cell anemia. Treatment of sickle erythrocytes with reagents that increase cAMP activates erythrocyte BCAM/Lutheran protein for some patients, allowing it to bind to the endothelial matrix protein laminin (Hines et al., 2003). cAMP appears to act through activation of Rap 1, a small guanosine triphosphatase that promotes integrin-mediated adhesion (Murphy *et al.*, 2004), and results in phosphorylation of Lutheran glycoproteins (Gauthier *et al.*, 2005). cAMP-mediated signaling in sickle erythrocytes also induces serine phosphorylation of ICAM-4, which increases erythrocyte adhesion to endothelial $\alpha_V\beta_3$ (Zennadi et al., 2004).

In addition to regulating transcription and expression of endothelial receptors and erythrocyte ligands, elevated cAMP also causes vasodilation in rabbit spinal arterioles (Yashiro & Ohhashi, 2002) and transgenic sickle mice (Kaul et al., 2000). As vasodilation is a vascular response generally attributed to production of endothelial nitric oxide (NO), subsequent studies revealed that cAMP elevating agents also increase NO concentration (X. Zhang & Hintze, 2001; X. P. Zhang *et al.*, 2002). cAMP induces

transcription of both endothelial and inducible nitric oxide synthases (eNOS and iNOS, respectively), the enzymes responsible for producing NO (Galea & Feinstein, 1999; Niwano *et al.*, 2003). Additionally, cAMP-dependent protein kinase (PKA) activates eNOS by phosporylating Serine-1177 (Michell *et al.*, 2001). The production of cAMP and NO are closely linked, as increasing NO has also been reported to increase cAMP in platelets (Aktas *et al.*, 2003), and NO shows much of the same potential as cAMP for regulating inflammatory gene expression (De Caterina et al., 1995; Khan *et al.*, 1996; Spiecker *et al.*, 1997). The role of NO in vascular biology and sickle cell anemia is outlined in the following section.

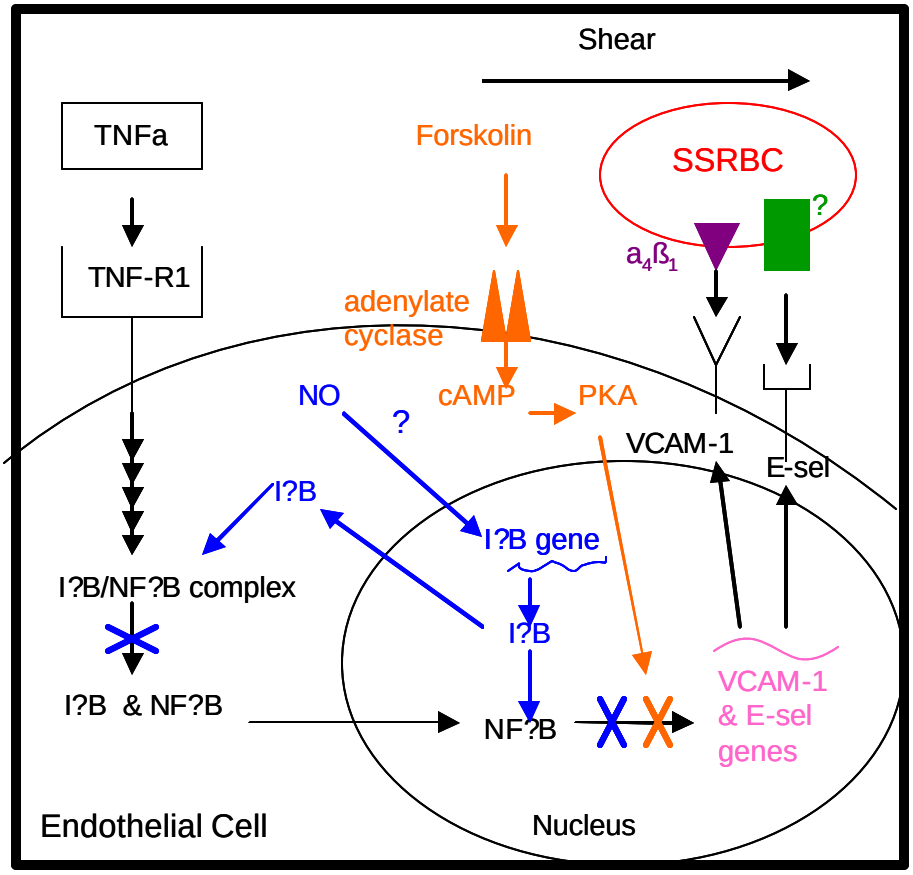


Figure 2.2 Intracellular cAMP and nitric oxide signaling in cytokine-induced sickle cell adherence. Forskolin (orange pathway) increases endothelial cAMP, which inhibits NF-κB from binding to its recognition sequence on VCAM-1 and E-selectin genes. Nitric oxide (blue pathway) inhibits VCAM-1 expression by increasing the expression and nuclear translocation of IκB.

2.7 The Role of Nitric Oxide in Sickle Cell Anemia and Endothelial Biology

Nitric oxide is a soluble gas synthesized in endothelial cells upon activation of the family of nitric oxide synthase enzymes. One of its primary physiological roles is the regulation of vascular tone. Once synthesized, NO is released from the endothelium as a gas or attached to transport molecules and activates soluble guanylyl cylase in smooth muscle cells, resulting in increased cyclic guanosine monophosphate (cGMP) in smooth muscle cells. The cGMP activates GMP-dependent kinases that decrease intracellular calcium concentrations, producing overall muscle relaxation and vasodilation (Gladwin & Schechter, 2001).

In addition to the important vasodilatory actions of NO, the molecule provides several other potentially beneficial effects to sickle patients. These effects, recently summarized by Gladwin and Schechter (Gladwin & Schechter, 2001), are wide in scope and target many of the elements of interest in sickle cell anemia:

- Inhaled NO acts on the pulmonary vasculature to reduce artery pressures, increase oxygenation, and improve ventilation.
- NO inhalation increases nitrosylated hemoglobin, which may affect oxygen affinity as well as delivery of NO to the peripheral vasculature.
- NO generated by hydroxyurea may contribute to the induction of fetal hemoglobin synthesis and reduced sickling of erythrocytes.
- NO may improve microvascular perfusion.
- NO inhibits platelet aggregation and may prevent thrombotic complications associated with sickle cell anemia.
- NO downregulates endothelial adhesion molecule expression.

Increased endothelial nitric oxide levels reduce cytokine-induced expression of the adhesion molecule VCAM-1 in a manner that appears independent of cGMP (De Caterina et al., 1995). Whether or not NO inhibits E-selectin and ICAM-1 expression is debatable (De Caterina et al., 1995; Khan et al., 1996). Inhibition of adhesion molecule expression is paralleled by reduced adhesion of monocytes

(De Caterina et al., 1995). Others have shown that increasing nitric oxide can inhibit neutrophil migration (Elferink & VanUffelen, 1996), platelet aggregation (Geiger, 2001), and adhesion of *Plasmodium falciparum*-infected erythrocytes (Serirom *et al.*, 2003). Additionally, increased NO decreases adhesion of nonsickle erythrocytes to TNF-α stimulated endothelium and adhesion of sickle erythrocytes to unstimulated endothelium (Space *et al.*, 2000). Conversely, decreasing endothelial NO content induces expression of VCAM-1 on otherwise unstimulated endothelium (De Caterina et al., 1995), and increases adhesion of uninfected (Space et al., 2000) and *Plasmodium falciparum*-infected nonsickle erythrocytes (Serirom et al., 2003). Reduction of NO in neutrophils has also been shown to induce surface expression of the VCAM-1 binding ligand $\alpha_4\beta_1$, resulting in increased adhesion of neutrophils to fibronectin (Conran *et al.*, 2003).

The mechanisms by which NO inhibits VCAM-1 expression are complex and are not fully understood. NO has been shown to stabilize the NF-κB-IκBα complex, preventing dissociation, activation, and nuclear translocation of NF-κB. It also increases mRNA expression of IκBα (Peng *et al.*, 1995). Furthermore, NO increases the concentration of IκBα in the

nucleus, where it could displace NF-κB from its cognate DNA (Spiecker et al., 1997). Figure 2.2 summarizes potential mechanisms by which NO inhibits endothelial VCAM-1 expression.

2.8 Chapter Summary

Sickle cell anemia is caused by the homozygous inheritance of the sickle β hemoglobin gene. Symptoms of the conditions include reversible aggregation of hemoglobin inside erythrocytes upon deoxygenation, hemolytic anemia, and vaso-occlusion resulting in painful crises, acute chest syndrome, stroke, retinopathy, priapism, and other complications. In sickle patients, both endothelial cells and erythrocytes exhibit abnormalities that likely contribute to clinical symptoms. The endothelium of sickle patients shows dimished capacity to regulate vascular tone and is also chronically activated to express proteins and receptors involved in coagulation and inflammation. One of the most important sickle erythrocyte abnormalities is increased adhesion to endothelial cells and endothelial matrix proteins, which likely initiates or propagates vaso-occlusion. Strategies to inhibit abnormal erythrocyte

adhesion to endothelial cells may benefit sickle patients, but current therapies for sickle cell anemia patients do not directly address adhesion. Since inflammation leads to endothelial expression of receptors VCAM-1 and E-selectin and sickle erythrocyte adhesion, anti-inflammatory reagents may help control adhesion and clinical complications in sickle cell anemia. Cyclic AMP and nitric oxide are two signaling molecules with anti-inflammatory properties. The ability of these molecules to prevent receptor expression and sickle erythrocyte adhesion is the subject of the following chapters.

CHAPTER 3

MATERIALS AND METHODS

3.1 Endothelial Cells

3.1.1 Obtaining Cells

Human dermal microvascular endothelial cells (MECs) were purchased at 2nd or 3rd passage from the Department of Dermatology at Emory University School of Medicine. Cells were received as frozen cell suspensions in cryovials or as confluent monolayers grown on T75 flasks.

Frozen cell suspensions were thawed in a water bath at 37°C, the cell suspension was centrifuged at 100g for 10 minutes, the supernatant was removed, and the cells were resuspended in 15 mL microvascular endothelial growth media (EGM-MV) purchased as a proprietary formula from Cambrex. Hydrocortisone supplements provided by Cambrex as part of the media formulation were not added to the media to avoid endothelial cell activation. The cells in media were placed in a T75 flask (Corning) coated overnight with 0.1%

porcine gelatin (Sigma) in Dulbecco's phosphate-buffered saline (DPBS, Sigma) and incubated at 37°C until cells reached confluency. Media was changed every 48 hours.

Human umbilical vein endothelial cells (HUVECs) were purchased at 3rd passage as confluent monolayers in T75 flasks from the Department of Dermatology at Emory University School of Medicine. All cells were passaged as described below.

3.1.2 Passaging

All endothelial cells were sterilly passaged by trypsin digestion. For MECs, confluent monolayers were washed two times with 6 mL MCDB131 (Media Tech) to remove any serum-containing growth media. After rinsing with MCDB 131, cells were quickly rinsed with 3 mL 0.05% trypsin/EDTA (Gibco) at 37°C, then treated with another 4 mL trypsin/EDTA. The flasks were gently tapped and observed under light microscopy to ensure cell detachment. To inactivate the trypsin, 6 mL EGM-MV was added to the flask. The cell suspension was removed from the flask and centrifuged at 100g for 10 minutes. The cell pellet was resuspended in EGM-MV and split 1:3. That is, the cells were placed in 3 gelatin-coated T75 flasks for further culture. The passage number was increased by one after

this and subsequent trypsin digestions. Cells were cultured at 37°C in a 95% air and 5% CO_2 incubator. Passaging of HUVECs was similar, but M199 (Sigma) was used instead of MCDB131 and HUVEC media was used instead of EGM-MV. HUVEC media consisted of M199 supplemented with 100 µg/mL heparin (Sigma), 20% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals), 2mmol/L L-glutamine (Sigma), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma), and 25 mg/mL endothelial cell growth factor (ECGF, Roche).

3.1.3 Storage

For storage, endothelial cells at passage 5 or 6 were passaged as described above. After centrifugation, cells were resuspended in 3 mL freezing solution rather than EGM-MV. Freezing solution consisted of 10% dimethylsulfoxide (DMSO, Sigma) in FBS. Three cryovials were each filled with 1 mL of the freezing solution. The vials were placed in a cell freezing container with an isopropanol refrigerant jacket which was placed in a -70°C or -80°C freezer for 24 hrs. The cells were then transferred to liquid nitrogen for long-term storage.

When cells were needed for experiments, one cryovial was unthawed and allowed to grow to confluency on T75

flasks as described in Section 3.1.1. After reaching confluency, cells were passaged as described in the next section for use in experiments.

3.1.4 Cell Culture for Use in Experiments

For all experiments, frozen MECs were thawed and grown to confluency on T75 flasks as described in Section 3.1.3. For flow assays, the confluent T75 was then passaged as described in Section 3.1.2, but after centrifugation, the cell pellet was resuspended in 32 mL EGM-MV. Two mL of the cell suspension was then placed in a single-well Nunc Permanox Lab Tek chamber slide that had been coated overnight with 5 µg/mL fibronectin (Sigma) in DPBS. Thus up to 16 slides for flow adhesion assays could be prepared from one confluent T75. The chamber slides were then tilted to one side by propping one end of each slide on a 1 mL pipette. After 6 minutes, the other end of the slide was propped up by the pipette for 6 minutes. This tilting allowed media to cover only half the chamber slide at one time and allowed for more even distribution of the endothelial cells over the surface of the chamber slide. Failure to tilt resulted in congregation of the MECs toward the middle of the slide, with many fewer cells attaching to the ends of the slides. Cells grew to confluency in 2 to 3

days. EGM-MV media was exchanged every 48 hrs during growth.

For ELISA experiments, 96-well tissue culture coated plates (Corning) were coated overnight with 0.1% porcine gelatin in DPBS. The available surface area for tissue culture for a single 96-well plate was either 30 cm^2 or 15 cm^2, depending on whether a full area or a half-area plate was used. To seed full area plates, a confluent T75 of MECs or HUVECs was passaged as described in Section 3.1.2. After centrifugation, the cell pellet was resuspended in 3 mL of either EGM-MV (MECs) or HUVEC growth media (HUVECs). 1 mL of cell suspension was then placed in 30 mL of media. 100 µL of diluted cell suspension was placed in each well. For half-area plates, only 50 uL of cell suspension was placed in each well. Media was exchanged every 48 hrs and cells reached confluency after 3 to 4 days.

For cAMP assays, MECs were grown to confluency on T75 flasks and passaged as described in Section 3.1.2. After centrifugation, the cell pellet was resuspended in 36 mL EGM-MV. Cells were seeded onto 6-well tissue culture coated dishes (Corning) coated overnight with 0.1% gelatin in DPBS. Two mL of cell suspension was placed in each well. Media was exchanged every 48 hours and cells grew to confluency in 3 to 4 days.

For viability assays, MECs were grown to confluency on T75 flasks and passaged as described in Section 3.1.2. After centrifugation, the cell pellet was resuspended in 36 mL EGM-MV. Cells were seeded onto round glass coverslips (VWR) placed in 12-well dishes (Corning) and coated overnight with 0.1% gelatin in DPBS. One mL of cell suspension was placed in each well. Media was exchanged every 48 hours and cells grew to confluency in 3 to 4 days.

3.2 Sickle Erythrocytes

Blood samples were collected by venipuncture from asymptomatic, homozygous sickle patients during regular visits to the Georgia Comprehensive Sickle Cell Center at Grady Memorial Hospital (Atlanta, GA) into heparin or EDTA-charged Vacutainer tubes. Patients did not show signs of infection or thromboembolic or liver disease, were not pregnant, and were not receiving hydroxyurea therapy. Protocols for drawing of blood were approved by the Institutional Review Boards of Emory University and Georgia Institute of Technology and the Research Oversight Committee at Grady Memorial Hospital. Experiments involving blood samples were completed between 2 to 56 from the time the blood was drawn.

To isolate erythrocytes, blood samples were centrifuged at 100g for 10 min at 25°C. The plasma layer and buffy coat were removed, and the erythrocyte-rich layer was resuspended to the original sample volume in DPBS supplemented with 0.2% (w/v) human albumin, 5 μg/mL human transferrin, and 5 μg/mL bovine insulin (all from Sigma). The washing process was repeated 2 more times. The hematocrit concentration of the red cell suspension was measured using a hematocytometer (Damon/IEC) and recorded. Samples were then stored at 4°C until 1 hr prior to use in flow assays.

3.3 Flow Adhesion Assays

3.3.1 System Description

A parallel plate flow chamber was used to quantify sickle erythrocyte adhesion to MECs. A diagram of the flow chamber and the assay system is given in Figure 3.1. Panel B shows the components of the actual flow chamber. The bottom plate of the chamber consisted of a confluent monolayer of MECs on a Lab-Tek Permanox plate. The bottom plate was separated from the top one by a plastic gasket of 0.004 in. thickness (Precision Brand, Downers Grove, IL). The inside perimeter of the gasket measured 1 cm by 4 cm.

The top plate of the chamber consisted of a polypropylene block with holes to allow flow of a sickle erythrocyte suspension between the two plates. The bottom plate, gasket, and top plate were held together by a thin layer of vacuum grease (Dow Corning). For additional stability, the plates were encompassed by aluminum brackets that were screwed tightly together.

To assemble the flow chamber confluent monolayers of MECs on Permanox Lab-Tek chamber slides were rinsed twice with 2 mL MCDB131 then covered with 1 mL fresh EGM-MV. The walls of the chamber were removed, and the slide was placed on top of the gasket and the polypropylene top plate. The holes in the top plate were filled with EGM-MV prior to flow chamber assembly to keep air out of the system. The flow chamber assembly was inverted and secured with the aluminum brackets. Tubing was used to connect the flow chamber entrance to a reservoir of serum-free media (SFM), consisting of MCDB 131 (GIBCO) containing 0.292 mg/mL L-glutamine (Sigma), 0.1 mg/mL each penicillin-streptomyocin (GIBCO), 5 µg/mL human transferrin, 5 µg/mL bovine insulin, 0.2% (w/v) human albumin, and 0.01 µg/mL endothelial growth factor (EGF, Clonetics). The exit port of the flow chamber was connected by tubing to a 60 mL syringe (Becton Dickinson), which when attached to a syringe pump (Harvard

Apparatus) drew media through the flow chamber (Figure 3.1, Panel A). The assembled flow chamber was placed on an inverted microscope (Nikon Diaphot, Tokyo, Japan), and the temperature of the chamber was maintained at 37°C by an air curtain incubator (Nicholson Precision Instruments, Bethesda, MD). The MECs were rinsed with SFM for 10 minutes prior to erythrocyte perfusion to remove proteins secreted by the endothelial cells. The temperature of the SFM and the erythrocyte suspension was maintained at 37°C by a water bath.

The shear stress at the walls of the flow chamber was maintained at 1 dyne/cm^2. This shear stress was chosen because it represents the typical wall shear stress in the post-capillary venules where occlusion is thought to occur *in vivo* (Goldsmith & Karino, 1987; Karino et al., 1987; Turitto, 1982). Shear stress in the flow chamber was determined using the momentum balance for fully developed Newtonian flow and parallel plate geometry (Bird *et al.*, 2002). The shear stress formula is as follows:

$$t_{wall} = \frac{6Qm}{bh^2}$$

where:

τ_{wall} = shear stress (1 dyne/cm^2)
Q = volumetric flow rate (0.171 mL/min)

μ = fluid viscosity (0.75 cpoise)
b = flow chamber width (1.0 cm)
h = flow chamber gap height (110 μm)

Fully developed Newtonian flow was assumed due to low Reynolds number and low hematocrit. Reynolds number was determined to be 0.76 by the following equation:

$$\mathrm{Re} = \frac{2h\nu r}{m} = \frac{2Qr}{bm}$$

where:

Re = Reynolds number (0.81)
h = flow chamber gap height (110 μm)
v = average velocity (m/s)
Q = volumetric flow rate (0.171 mL/min)
ρ = fluid density (1 g/cm^3)
b = flow chamber width (1.0 cm)
μ = fluid viscosity (0.75 cpoise)

After a 10 minute rinse with SFM at 1 dyne/cm^2 shear stress, sickle erythrocytes were perfused over MECs for 40 minutes. Sickle erythrocytes were washed as discussed in Section 3.2, then suspended at 0.22% hematocrit in 9 mL SFM. The number of firmly adherent sickle erythrocytes was counted in 20 random microscopic fields at 400x magnification at approximately 1, 3, 5, 10, 20, 30, and 40 minutes after initiation of erythrocyte perfusion. The number of adherent endothelial cells in a field was determined visually, then recorded by the experimenter in a Microsoft Excel spreadsheet. Macros were used to record

the clock time each data point was taken and the time after initiation of erythrocyte perfusion. Macros are given in Appendix A. Results at each time point were averaged and normalized to adherent erythrocytes per square millimeter of endothelium. Experiments were also recorded by a camera (CCD72, MTI) attached to a television (Trinitron) and video cassette recorder (JVC).

For some experiments MECs or sickle erythrocytes were treated prior to the flow adhesion assay. Treatments are described in Sections 3.3.2 and 3.3.3.

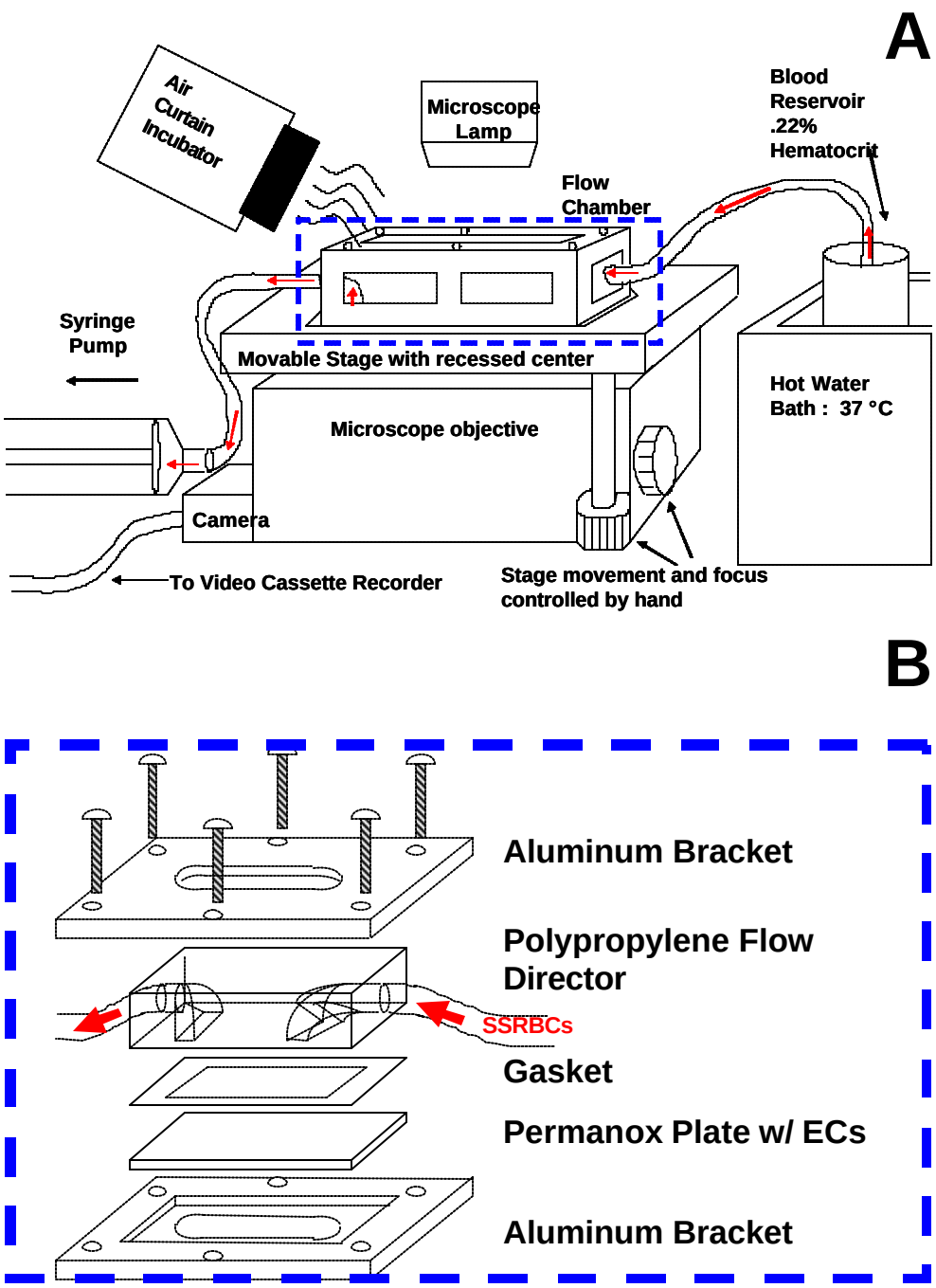


Figure 3.1 Flow adhesion assay system. A) A parallel plate flow chamber mounted on a microscope attached to a camera, TV, and VCR. A syringe pump draws an erythrocyte suspension through the flow chamber. The system is warmed by a water bath and an air curtain incubator. B) Components of the parallel plate flow chamber.

3.3.2 Endothelial Treatments

To test mechanisms of binding of sickle erythrocytes to endothelium, MECs were stimulated with TNF-α (500 U/mL) for 2 or 6 hrs prior to sickle erythrocyte perfusion. Some MECs were simultaneously treated with monoclonal antibodies to block adhesion molecules and inhibit sickle erythrocyte adhesion. Antibodies for adhesion molecules of interest included anti-VCAM-1 antibody (20 μg/mL, Beckman Coulter) and anti-E-selectin antibody (30 μg/mL, Beckman Coulter). Antibody concentrations used were chosen due to their effectiveness in similar experiments in literature and also due to practical limits of expense. To test for antibody and adhesion molecule specificity, control antibodies that were not expected to inhibit sickle erythrocyte adhesion were also used. These included anti-ICAM-1 antibody (20 μg/mL, BD Bioscience) and anti-IgGκ antibody (30 μg/mL, Sigma).

In addition to monoclonal antibodies, a synthetic peptide (100 μM; higher concentrations were not used due to solubility limits) was also used to block E-selectin and inhibit sickle erythrocyte adhesion. The peptide sequence was H_2N-DITWDQLWDLMK-COOH and was previously shown to inhibit neutrophil adhesion to E-selectin (Martens *et al.*, 1995). In experiments using the synthetic peptide, the

peptide was added not only to the endothelial media solution during TNF-α stimulation, but also to the sickle erythrocyte suspension to ensure continuous availability of the peptide during flow. To control for the addition of peptide to the erythrocyte suspension, in some experiments peptide was added to the erythrocyte suspension for 40 minutes, then erythrocytes were centrifuged at 100g for 10 minutes and resuspended in peptide-free SFM. Also, to control for peptide specificity, a scrambled version of the peptide (H_2N-QLITWDMDDKWL-COOH) was used.

To test the ability of cAMP to regulate adhesion molecule expression and sickle erythrocyte adhesion, some endothelial monolayers were treated with 100 μM forskolin (Fsk), 500 μM isobutylmethylxanthine (IBMX), or 500 μM dibutyryladenosine 3’5’-cyclic monophosphate (dibutyryl cAMP or Bt_2cAMP, all from Sigma) for 30 minutes prior to and during 6 hrs TNF-α stimulation to elevate cAMP levels. In addition, some endothelial monolayers were pretreated with 100 μM Fsk, then stimulated with TNF-α alone (without Fsk) for 2 or 6 hrs. The negative controls for all experiments were adhesion of sickle erythrocytes to unstimulated MECs and adhesion to MECs treated with Fsk, IBMX, or Bt_2cAMP alone for 30 minutes or 6.5 hrs.

To test the ability of NO to regulate adhesion molecule expression and sickle erythrocyte adhesion, some endothelial monolayers were treated with 500 μM sodium nitroprusside (SNP, Sigma) or 500 μM 2,2'-(Hydroxynitrosohydrazono)bis-ethanimine (DETA-NO, Sigma) to increase endothelial NO content for 30 minutes prior to and during 6 hrs of TNF-α stimulation. Controls for all experiments were adhesion of sickle erythrocytes to unstimulated MECs and adhesion to MECs treated with SNP or DETA-NO alone for 6.5 hrs.

3.3.3 Sickle Erythrocyte Treatments

To test for binding ligands on sickle erythrocytes, erythrocytes were treated with anti-α_4 antibody (50 μg/mL, Beckman Coulter) to block erythrocyte $\alpha_4\beta_1$ or neuraminidase (300 μM Beckman Coulter) to cleave sialic acid residues for 40 minutes, centrifuged at 100g for 10 minutes, and resuspended in SFM prior to perfusion over MECs stimulated with TNF-α for 6 hrs.

To test the effects of reagents that affect erythrocyte cAMP or NO concentrations on adhesion of sickle erythrocytes, sickle erythrocytes were incubated with 100 μM Fsk, 500 μM IBMX, 500 μM Bt_2cAMP, 500 μM SNP, or 500 μM

DETA-NO for 40 minutes, centrifuged at 100g for 10 minutes, and resuspended in SFM prior to perfusion over unstimulated MECs or MECs stimulated with TNF-α for 6 hrs.

3.4 Enzyme Linked Immunosorbant Assay

To test the effects of cytokine stimulation on endothelial adhesion molecule expression, confluent monolayers of MECs and HUVECs in 96 well dishes were stimulated with TNF-α (500 U/mL) for times ranging from 0 to 24 hrs. Enzyme linked immunosorbant assay (ELISA) was used to measure VCAM-1, E-selectin, and ICAM-1 expression as described below.

To test the effects of reagents that alter cAMP or NO concentrations on endothelial adhesion molecule expression, MECs were pretreated with Fsk, IBMX, Bt_2cAMP, SNP, or DETA-NO, then stimulated with TNF-α plus or minus the aforementioned reagents for 2 or 6 hrs. Pretreatment times ranged from 0 to 2 hrs and concentrations of reagents used varied from 0 to 1000 μM.

After treatment, all cells were fixed with 10% formalin (Fisher) and refrigerated overnight in 1% bovine serum albumin (BSA) in DPBS. Cells were then incubated for

1 hr with either anti-VCAM-1, anti-E-selectin, or anti-ICAM-1 primary antibody (1 μg/mL) in the BSA solution. Wells were then rinsed 3 times with BSA and treated with peroxidase conjugated sheep anti-mouse IgG secondary antibody (Sigma) at 1 μg/mL in BSA. Secondary antibody binding was detected by reaction of o-phenylenediamine dihydrochloride (OPD) with H_2O_2 (SIGMA FAST™ OPD tablets, Sigma) for 30 minutes. Wells treated with secondary antibody only served as controls for each plate. Optical density at 450 nm was measured by a Spectramax Plus 384 Microplate Reader (Molecular Devices).

3.5 cAMP Measurement

To correlate cAMP concentrations with adhesion molecule expression and sickle erythrocyte adhesion, confluent monolayers of MECs in 6-well dishes were pretreated with Fsk for 30 minutes, then stimulated with TNF-α with or without Fsk for times ranging from 0 to 6 hrs. Intracellular endothelial cAMP was measured using a Correlate-EIA™ Direct Cyclic AMP Enzyme Immunoassay Kit (AssayDesigns, Ann Arbor, MI). Confluent and appropriately stimulated endothelial cells were lysed by incubation in

0.1 M HCl, the lysate was centrifuged at 600g for 10 min, and supernatant was used in the assay. The kit used polyclonal antibody to cAMP to competitively bind either cAMP in the sample or cAMP molecules conjugated to alkaline phosphatase provided in the kit. 100 µL of sample, 50 µL of conjugated cAMP, and 50 µL cAMP antibody were added to individual wells of a 96-well dish coated with goat anti-rabbit IgG and shaken on a plate shaker at 500 rpm for 2 hours. Wells were washed with 200 µL of supplied wash solution 3 times, and then 200 µL of the supplied p-nitrophenyl phosphate substrate wass added to each well. After incubation for 1 hour, 50 µL of supplied stop solution was added to each well, and the plate was read at 405 nm on a Power Wave$_X$ 340 Microplate Reader. Comparison of optical density with that of provided standards allowed for quantification of cAMP in endothelial supernatant samples.

3.6 Cell Viability Assay

Viability of cell cultures was evaluated by visual inspection of endothelial monolayers by light microscopy and by a Live/Dead Viability/Cytotoxicity kit (Molecular Probes, Eugene, OR). For this assay, endothelial monolayers were rinsed in DPBS 3 times for 10 minutes each,

incubated with a dye solution at 37°C for 30 minutes, rinsed three more times in DPBS, and stored away from light until microscope viewing. Live cells were stained green with calcein AM dye (ex/em 495 nm/515 nm), and dead cells were stained red with ethidium homodimer-1 (ex/em 495 nm/635 nm). Fluorescence was detected by a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, NY).

3.7 Statistical Analysis

One-factor analysis of variance (ANOVA) was used to test differences between levels of adherence of sickle erythrocytes under various endothelial treatment conditions, between differences in levels of adhesion molecule expression under various endothelial treatment conditions, and differences in endothelial cAMP concentrations due to forskolin treatment. Fisher's pairwise comparisons were used to test for differences in individual treatment means when ANOVA yielded statistical difference. Analyses were completed using MINITAB™ Statistical Software.

CHAPTER 4

CELL ADHESION MOLECULES SUPPORTING ADHESION OF SICKLE ERYTHROCYTES TO TNF-a STIMULATED ENDOTHELIUM

As discussed in more detail in section 2.2.3, vascular occlusion is a cause of many of the clinical manifestations associated with sickle cell anemia. The abnormal adherence of sickle erythrocytes to the endothelium likely contributes to occlusive events; however, mechanisms of adhesion are still under investigation. Several previous studies have demonstrated increased sickle erythrocyte adherence to cytokine-stimulated endothelium, most of which used cultured HUVECs, or large vessel endothelial cells, as a representative endothelial model. Occlusion, however, often occurs in the microcirculation where endothelial cells are phenotypically different (H. A. Brittain et al., 1992; Swerlick *et al.*, 1992). The following studies compare adhesion molecule expression on microvascular endothelial cells (MECs) with that of HUVECs and

demonstrate mechanisms of sickle erythrocyte adhesion to MECs.

4.1 Endothelial Cell Adhesion Molecule Expression

4.1.1 TNF-α Stimulated Microvascular Endothelial Cells

Stimulation of endothelial cells with cytokines such as TNF-α induces expression of VCAM-1, E-selectin, and ICAM-1, although expression profiles with increased stimulation time may be different depending on endothelial cell phenotype. ELISA was used to measure expression of these receptors on MECs. Results are shown in Figure 4.1.

Panel A of Figure 4.1 shows minimal expression of VCAM-1 on unstimulated MECs, and expression is not significantly upregulated until 6 hrs of TNF-α stimulation. This delay is consistent with reports in literature for this cell type (Swerlick et al., 1992). E-selectin expression, however, is significantly increased after only two hours of TNF-α stimulation (Panel B). Unlike VCAM-1 and E-selectin, ICAM-1 is constitutively expressed on unstimulated MECs, and TNF-α stimulation for 4 or more hours further increases expression (Panel C).

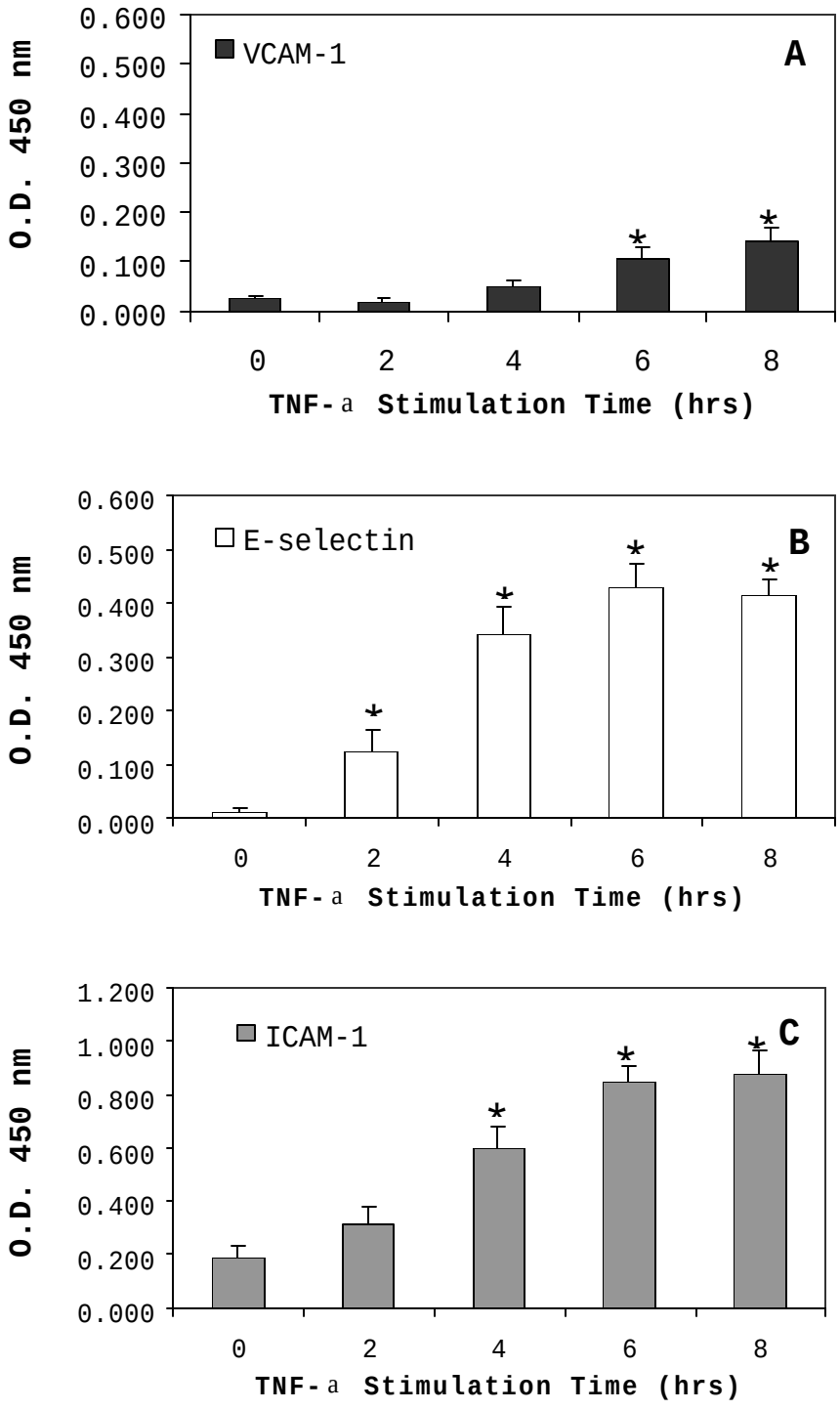


Figure 4.1 Adhesion molecule expression on MECs. A) VCAM-1, B) E-selectin, and C) ICAM-1 expression on MECs as a function of TNF-α (500 U/mL) stimulation time. *Indicates significant difference from 0 hrs stimulation (n=9). Data for individual experiments are given in Table B.1.

4.1.2 Comparison of Adhesion Molecule Expression on MECs and HUVECs

To determine phenotypic differences in expression of adhesion molecules, expression of VCAM-1, E-selectin, and ICAM-1 was measured by ELISA for up to 24 hrs of TNF-α stimulation. Figure 4.2 shows results for VCAM-1. For MECs, upregulation of VCAM-1 is apparent after 6 hrs of TNF-α stimulation and continues to increase until it peaks at 16 hrs (Panel A). After this time, VCAM-1 expression begins to decrease, consistent with literature (Swerlick et al., 1992). VCAM-1 expression is increased on HUVECs after 6 hrs of stimulation; however, expression then plateaus until 20 hrs of stimulation (Panel B). At 24 hrs of stimulation, expression begins to increase again.

E-selectin expression profiles are similar between MECs and HUVECs (Figure 4.3), but differ from those of VCAM-1 for either cell type. Upregulation of E-selectin is evident after only 2 hrs of TNF-α stimulation, but expression peaks at 6 hrs (HUVECs) or 8 hrs (MECs). After this, surface expression declines steadily but does not reach baseline (expression levels on unstimulated cells) after 24 hrs of stimulation.

ICAM-1 expression profiles are shown in Figure 4.4. Both MECs and HUVECs express ICAM-1 under unstimulated conditions, but HUVECs demonstrate higher optical densities than MECs. Expression of ICAM-1 on MECs increases sharply for the first 12 hrs of TNF-α stimulation, then plateaus out to 24 hrs (Panel A). On HUVECs, ICAM-1 expression is high on unstimulated cells and increases less sharply in response to TNF-α stimulation than MECs (Panel B).

Together these data demonstrate phenotypic differences between HUVECs and MECs with regard to TNF-α stimulated adhesion molecule expression. These differences suggest that the choice of an endothelial model for *in vitro* flow studies of sickle erythrocytes needs to be undertaken with care and attention to mimicking as closely as possible the *in vivo* environment one wishes to study. Because many of the clinical manifestations of sickle cell anemia result from microvascular occlusion, microvascular endothelial cells are used for all further studies.

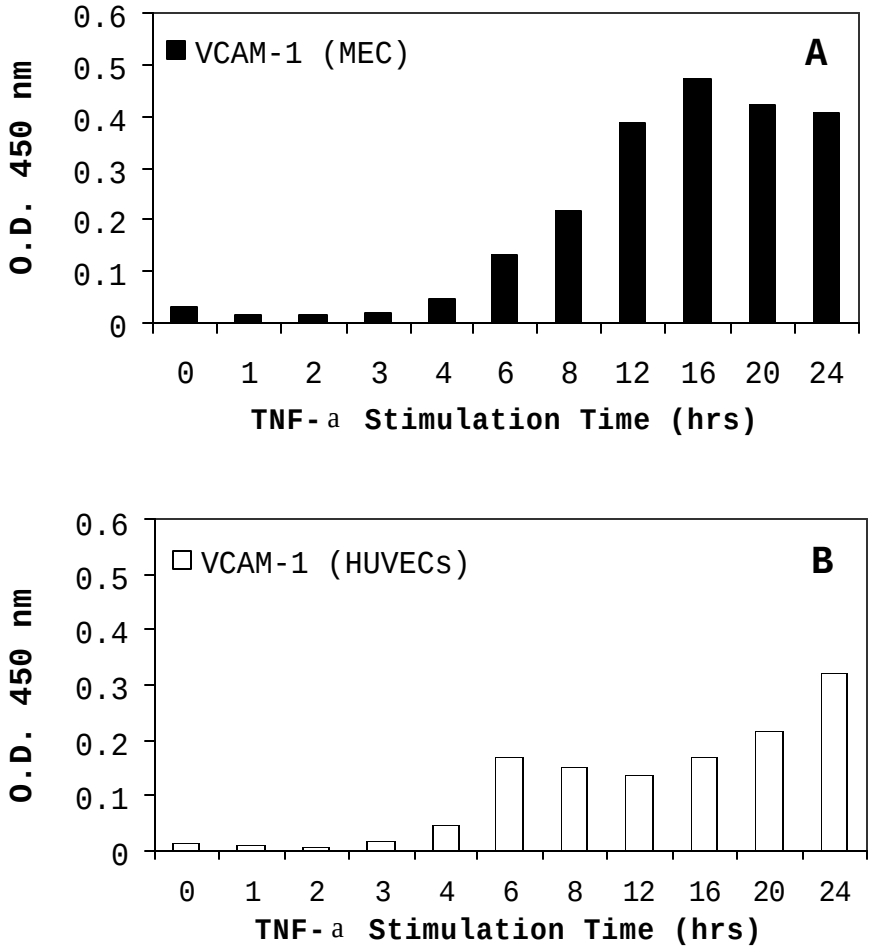


Figure 4.2 VCAM-1 expression on MECs and HUVECs. VCAM-1 expression on MECs (A) and HUVECs (B) as a function of TNF-α stimulation time (n=2). Data from individual experiments are given in Table B.3.

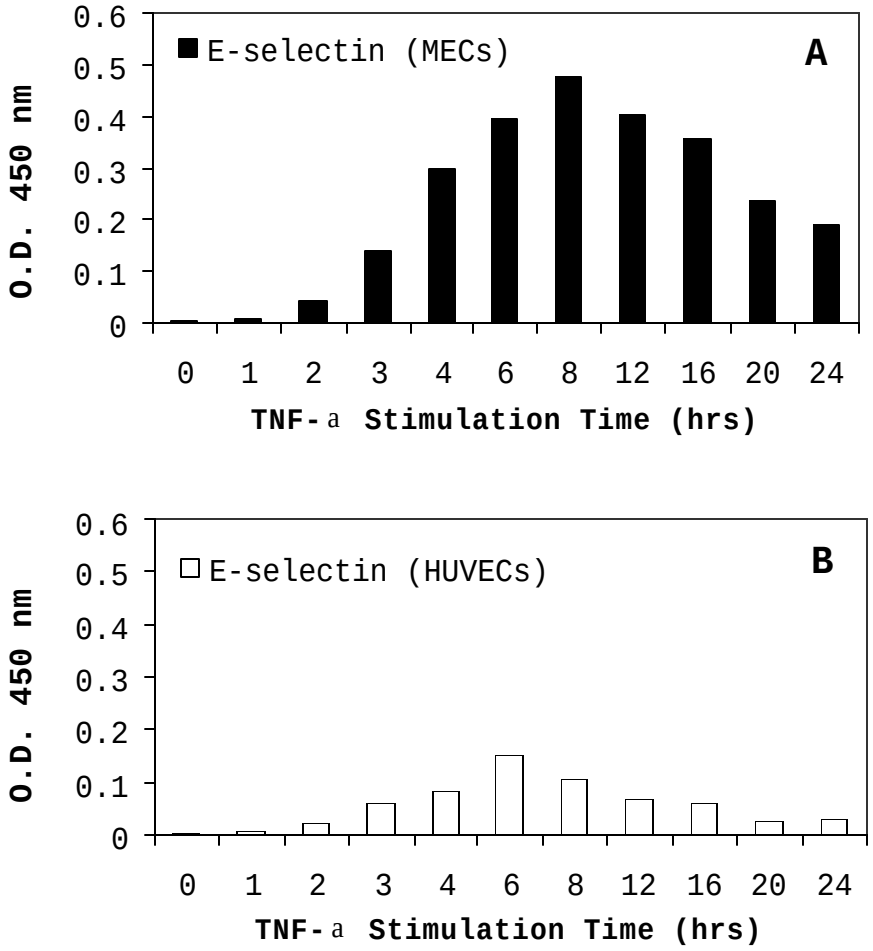


Figure 4.3 E-selectin expression on MECs and HUVECs. E-selectin expression on MECs (A) and HUVECs (B) as a function of TNF-α stimulation time (n=2). Data from individual experiments are given in Table B.4.

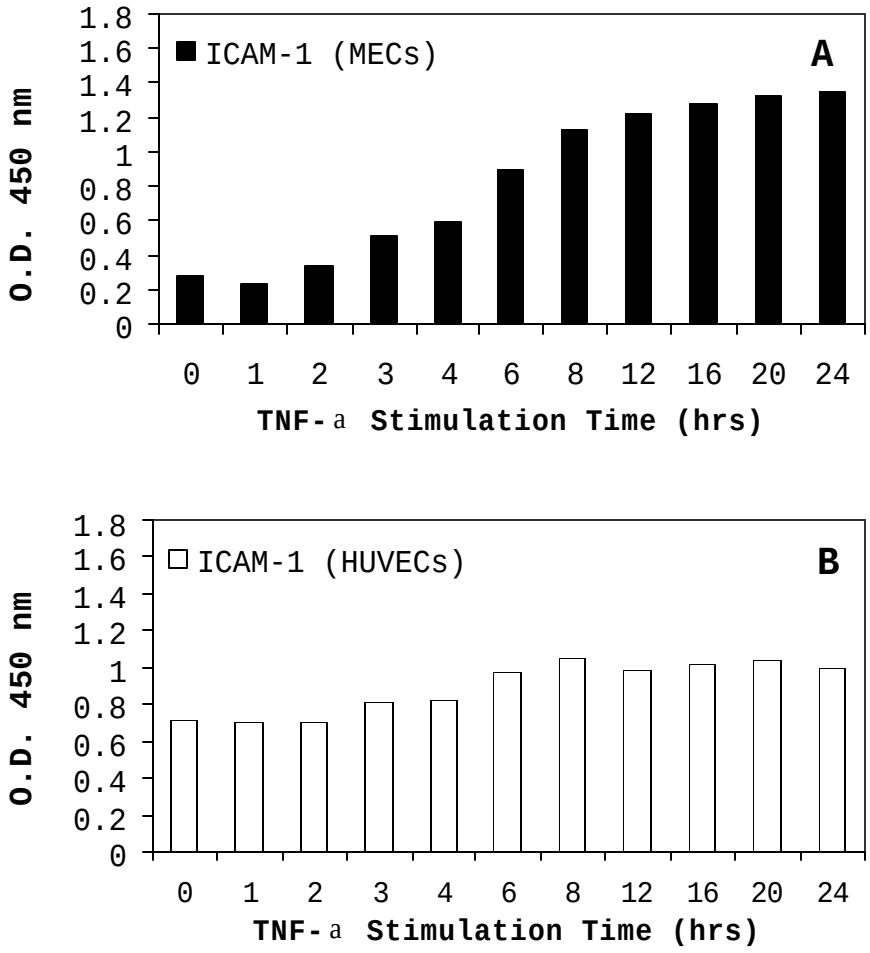


Figure 4.4 ICAM-1 expression on MECs and HUVECs. ICAM-1 expression on MECs (A) and HUVECs (B) as a function of TNF-α stimulation time (n=2). Data from individual experiments are given in Table B.5.

4.2 Sickle Erythrocyte Adhesion to TNF-α Stimulated MECs

Increased endothelial adhesion molecule expression due to TNF-α stimulation suggests that adhesion of sickle erythrocytes increases in response to such stimulation. To test this hypothesis, MECs were stimulated with TNF-α for either 2 or 6 hours. Sickle erythrocytes were perfused over the MECs at 1 dyne/cm^2 shear stress and adherent erythrocytes counted for up to 40 minutes of perfusion (see Section 3.3 for more details).

Results are shown in Figure 4.5 below. Adhesion of sickle erythrocytes to unstimulated MECs is minimal, reaching only 7.0 ± 2.8 cells/mm^2 after 40 minutes of perfusion. Stimulation of MECs with TNF-α for 2 hrs increases adhesion to 18 ± 1.9 cells/mm^2; however, this increase does not achieve statistical significance. Stimulation of MECs with TNF-α for 6 hrs further increases adhesion to 44 ± 8.0 cells/mm^2. This value is significantly higher than that for both unstimulated MECs and those stimulated with TNF-α for 2 hrs. For both 2 and 6 hrs of TNF-α stimulation, sickle erythrocyte adhesion increases sharply at short erythrocyte perfusion times, then reaches a plateau after 30 to 40 minutes of perfusion.

Comparing Figure 4.1 and Figure 4.5 suggests possible mechanisms of sickle erythrocyte adhesion. Unstimulated MECs express only ICAM-1 (not VCAM-1 or E-selectin), indicating that adherent erythrocytes might be bound to ICAM-1. However, since only small numbers of sickle erythrocytes adhere to unstimulated MECs it is unlikely that ICAM-1 supports adhesion of sickle erythrocytes. That ICAM-1 is not involved in sickle erythrocyte adhesion is confirmed by others (Montes *et al.*, 2002) and by antibody blocking studies presented in Section 4.3.3. After 2 hrs of TNF-α stimulation, E-selectin expression, but not VCAM-1 expression is upregulated. This suggests that sickle erythrocyte adhesion above levels on unstimulated MECs results from binding of sickle erythrocytes to E-selectin. After 6 hrs of TNF-α stimulation, expression of both VCAM-1 and E-selectin is upregulated, and the increase in sickle erythrocyte adhesion may be due to binding of erythrocytes to either E-selectin or VCAM-1. Mechanisms of adhesion of sickle erythrocytes to TNF-α stimulated MECs are further tested using monoclonal antibodies to block adhesion via ICAM-1, E-selectin, and VCAM-1 in the studies presented in Section 4.3.

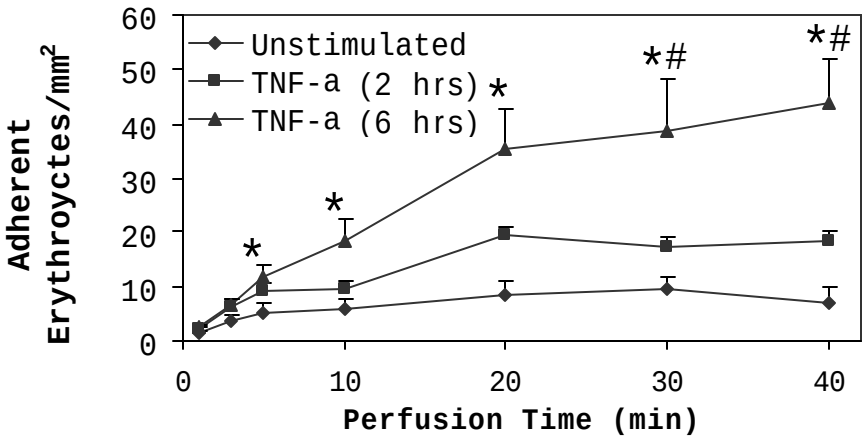


Figure 4.5 Adhesion of SSRBCs to TNF-a stimulated MECs. Sickle erythrocyte adhesion to MECs increases with TNF-α stimulation time(n=8). *Indicates statistical difference from unstimulated MECs. #Indicates statistical difference from MECs stimulated with TNF-α for 2 hrs. Data from individual blood samples are given in Table B.6.

4.3 Blocking of Adhesion Molecules with Monoclonal Antibodies

As mentioned in Section 4.2, mechanisms of sickle erythrocyte adhesion to MECs are elucidated by incubating endothelial cells with monoclonal antibodies against VCAM-1, E-selectin, or both during TNF-α stimulation. Antibodies specifically fill the binding pocket of the adhesion molecule against which they are raised (i.e. VCAM-1 or E-selectin), thereby preventing sickle erythrocytes

from binding to the endothelial cells via the adhesion molecule. Because monoclonal antibodies are relatively large and VCAM-1 and E-selectin are likely in close proximity on the endothelial surface, one concern in using monoclonal antibodies is that attachment of an antibody to one type of adhesion molecule might sterically hinder access of sickle erythrocytes to the other type. Thus to verify results obtained using monoclonal antibodies, some endothelial monolayers are also treated with a synthetic peptide (12 amino acids in length) which blocks E-selectin and is only about 1% of the size of a typical antibody, lessening the chance of sterically hindering VCAM-1. Because ICAM-1 is not expected to support sickle erythrocyte adhesion, only a select few experiments are completed using anti-ICAM-1 antibodies. They are presented as control data in Section 4.3.3. Antibody blocking experiments using anti-VCAM-1 and anti-E-selectin antibodies are completed for both 2 and 6 hrs of TNF-α stimulation and results are shown below.

4.3.1 Monoclonal Antibodies Blocking Adhesion Molecules on MECs Stimulated with TNF-a for 2 hrs

Panel A of Figure 4.6 shows that treatment of MECs with anti-VCAM-1 antibody does not inhibit sickle

erythrocyte adhesion on MECs stimulated with TNF-α for 2 hrs, suggesting that VCAM-1 does not support adhesion of sickle erythrocytes at this time.

While Panel A represents average data for 8 blood samples, individual samples varied greatly in levels of adhesion, thus resulting in large error bars. Adhesion to TNF-α stimulated MECs varied from 8 to 229 cells/mm^2 after 40 minutes of perfusion, depending on the blood sample (See Table B.7 for the complete data set). For clarity, Panel B of Figure 4.6 shows results from individual blood samples comprising the averages shown in Panel A at the 40 minute time point. Results are shown as fold change, where

$$Fold\ Change = \frac{\#\ of\ \ Adherent\ \ SSRBCs\ \ for\ Treatment\ Shown\ on\ x-axis}{\#\ of\ \ Adherent\ \ SSRBCs\ on\ Unstimulated\ \ MECs}$$

For the 8 blood samples tested, 6 showed a 1.5 fold or greater increase in adherent sickle erythrocytes due to 2 hrs of TNF-α stimulation. Treatment of MECs with anti-VCAM-1 antibody inhibited adhesion in only 2 of the samples (S38 and S45). Results from individual blood samples as well as the combined average indicate that VCAM-1 does not

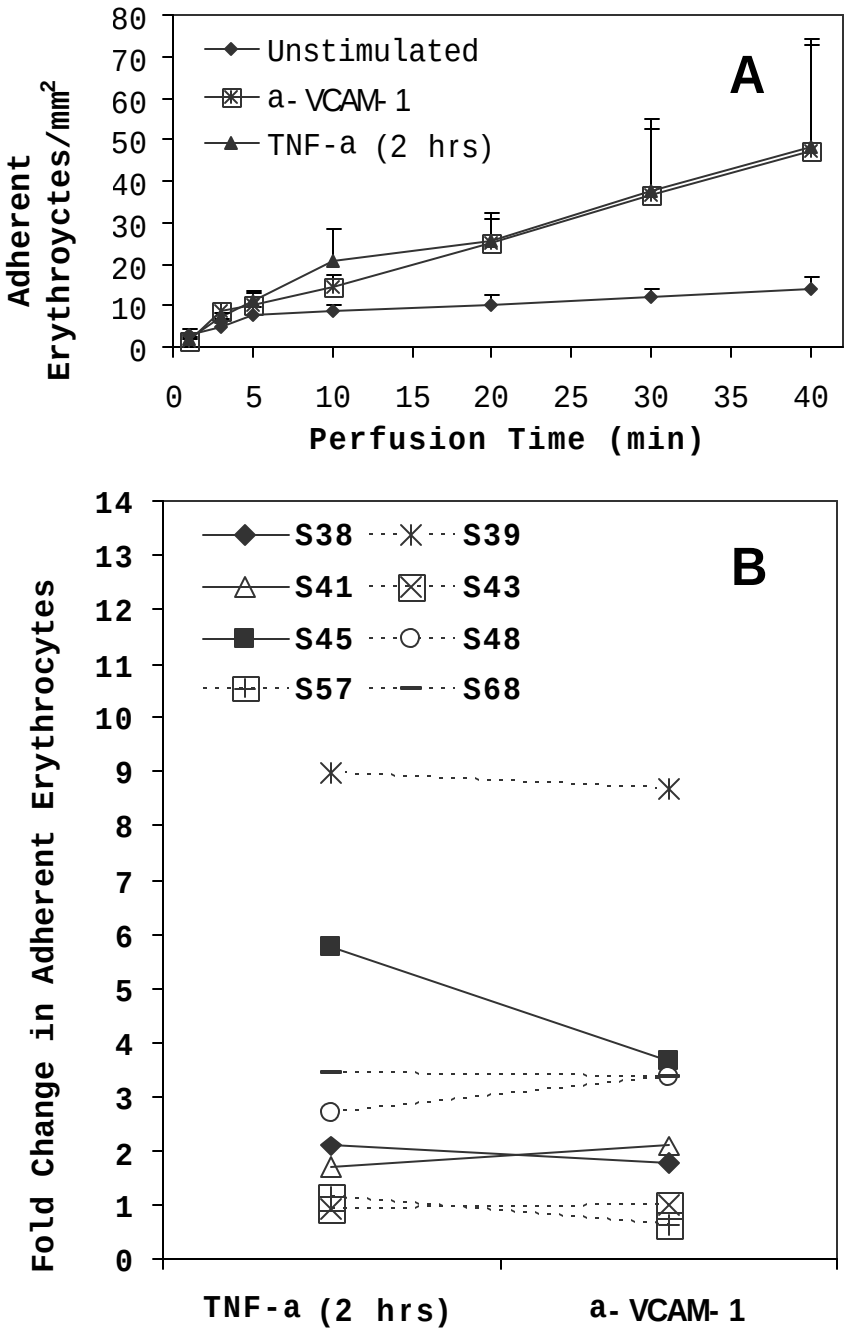


Figure 4.6 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 2 hrs by anti-VCAM-1 antibody. A) Average data for n=8 blood samples. B) Fold change in adhesion after 40 minutes of perfusion for individual blood samples comprising the average shown in Panel A. Data for individual blood samples are given in Tables B.7 and B.8.

promote adhesion of sickle erythrocytes to MECs after 2 hrs of TNF-α stimulation. These results are expected, since ELISA data shown in Figure 4.1 demonstrate that VCAM-1 is not upregulated on the endothelial cell surface after only 2 hrs of stimulation.

Figure 4.7 shows blocking of endothelial E-selectin. In Panel A, a small synthetic peptide which binds to E-selectin inhibits adhesion of sickle erythrocytes by 49%. Again, these results are not statistically significant due to large variability in absolute numbers of adhesive erythrocytes between blood samples. Panel B shows fold changes in adhesion for all blood samples tested after 40 minutes of perfusion. Of the 8 samples tested, 6 demonstrate a 1.5 fold or higher increase in sickle erythrocyte adhesion due to TNF-α stimulation. Of these 6, 5 show a decrease in sickle erythrocyte adhesion due to treatment with the E-selectin blocking peptide. For three samples, treatment of MECs with the peptide results in a fold change that is less than one, meaning that adhesion is less than that for unstimulated MECs. While not statistically significant, these results suggest that E-selectin may mediate adhesion of sickle erythrocytes to MECs in some patients. These results are further supported by data showing that treatment of MECs with anti-E-selectin

antibody reduces adhesion of sickle erythrocytes by 49% (Figure 4.8). Testing of a larger patient pool may help to clarify the role of E-selectin in promoting adhesion at this early TNF-α stimulation time.

Interesting to note is that blocking with either the E-selectin peptide or the E-selectin antibody does not completely inhibit adhesion of sickle erythrocytes. These data are consistent with the only other report of E-selectin mediated adhesion of sickle erythrocytes available in the literature to date (Natarajan *et al.*, 1996). One potential explanation for incomplete inhibition of adhesion is that too little reagent (either peptide or antibody) was used. More peptide could not be used due to solubility limits of the peptide in media. More antibody was not used due to practical limits of expense. Another potential explanation for incomplete inhibition is that an unknown adhesion molecule other than E-selectin promotes sickle erythrocyte adhesion. However, this seems unlikely given the current body of literature suggesting that the primary adhesion molecules upregulated by TNF-α stimulation are VCAM-1, E-selectin, and ICAM-1.

Also important to note is that treatment of MECS with both the E-selectin blocking peptide and anti-VCAM-1 antibody simultaneously inhibits adhesion of sickle

erythrocytes by 71%, or 22% more than blocking with the E-selectin peptide alone. For the given data set, these results are not statistically different; however, they invite speculation as to how the antibody and the peptide might interact with one another when VCAM-1 is not present on the endothelial cell surface and how their interaction might affect experimental outcomes. To date, no studies about such interactions are available in the literature.

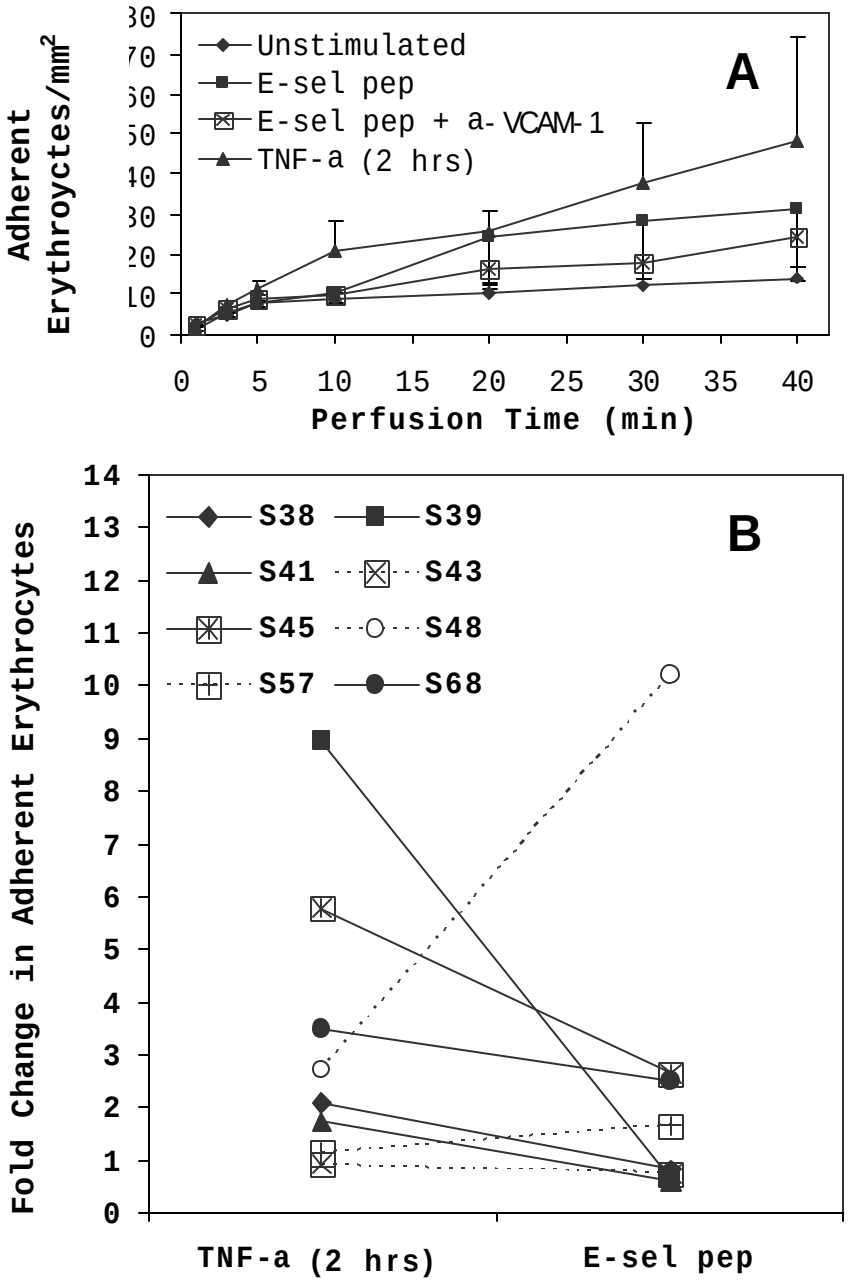


Figure 4.7 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 2 hrs by E-selectin blocking peptide and anti-VCAM-1 antibody. A) Average data for n=8 blood samples. B) Fold change in adhesion after 40 minutes of perfusion for individual blood samples comprising the average shown in Panel A. Data for individual blood samples are given in Tables B.9 and B.10.

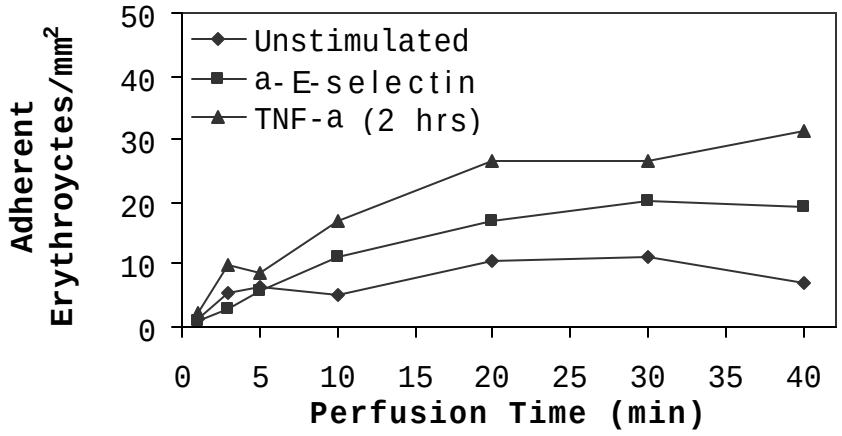


Figure 4.8 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 2 hrs by anti-E-selectin antibody. Average data for n=2 blood samples. Data for individual blood samples are given in Table B.11.

4.3.2 Monoclonal Antibodies Blocking Adhesion Molecules on MECs Stimulated with TNF-a for 6 hrs

After 6 hrs of TNF-α stimulation, both VCAM-1 and E-selectin are upregulated on MECs (Figure 4.1). Blocking studies using antibodies and peptides are conducted to determine which adhesion molecules support sickle erythrocyte adhesion. Panel A of Figure 4.9 shows that treatment of MECs with anti-VCAM-1 antibody inhibits adhesion of sickle erythrocytes by 72%, suggesting that VCAM-1 is a dominant adhesion molecule supporting sickle erythrocyte adhesion after 6 hrs of TNF-α stimulation. Blocking with the E-selectin blocking peptide inhibits

sickle erythrocyte adhesion by 59%, while blocking with an anti-E-selectin antibody inhibits adhesion by 67%, demonstrating that E-selectin also contributes to sickle erythrocyte adhesion (Figure 4.9, Panels B and C). Treatment of MECs with both anti-VCAM-1 antibody and E-selectin blocking peptide simultaneously inhibits adhesion of sickle erythrocytes 82% (Figure 4.9, Panel B).

Together these data demonstrate that both VCAM-1 and E-selectin support sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs. Blocking both adhesion molecules together more effectively inhibits sickle erythrocyte adhesion than blocking either VCAM-1 or E-selectin alone, suggesting that adhesion via both molecules may need to be controlled to elicit *in vivo*, clinical effectiveness.

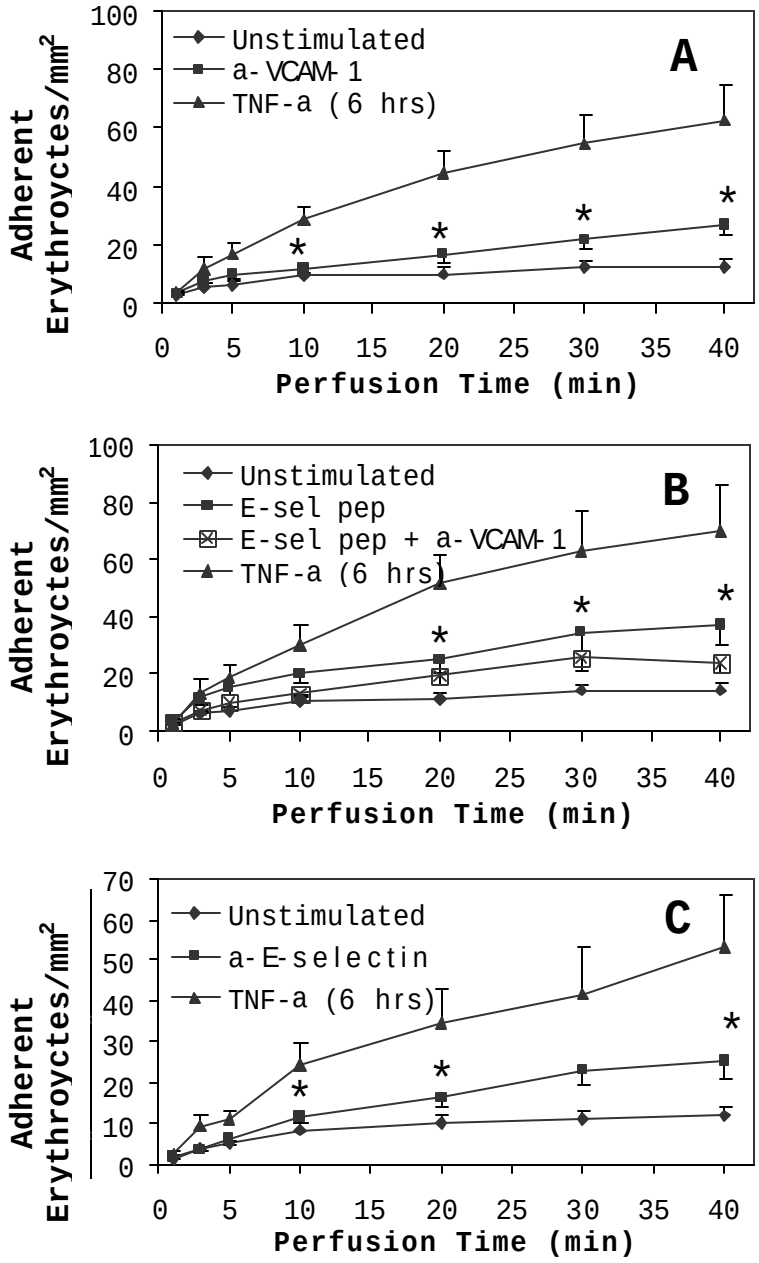


Figure 4.9 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs. Blocking with A) anti-VCAM-1 antibody (n=13), B) E-selectin blocking peptide and/or anti-VCAM-1 antibody (n=9), and C) anti-E-selectin antibody (n=10). *Indicates statistical difference from TNF-a (6 hrs). For Panel B, statistical significance is indicated for both E-sel pep and E-sel pep + a-VCAM-1. Data for individual blood samples are given in Tables B.12 through B.14.

4.3.3 Control Studies with Non-blocking Antibodies and Peptides

To ensure that the effectiveness of VCAM-1 and E-selectin antibodies at inhibiting sickle erythrocyte adhesion is due to specific binding of the antibodies to the adhesion molecule against which they were raised and not due to non-specific binding, MECs stimulated with TNF-α for 6 hrs are treated with either anti-ICAM-1 antibody or anti-IgGκ antibody, then perfused with sickle erythrocytes as previously described. As mentioned earlier in Section 4.3, ICAM-1 is a specific adhesion molecule not thought to support adhesion of sickle erythrocytes. The IgGκ antibody is a non-specific antibody. The results shown in Figure 4.10 show that treatment with either anti-ICAM-1 antibody or anti-IgGκ antibody does not inhibit sickle erythrocyte adhesion, suggesting that inhibition of adhesion due to treatment with anti-VCAM-1 or anti-E-selectin antibodies is due to specific inhibition of VCAM-1 and E-selectin on MECs.

Control experiments are also performed for the E-selectin blocking peptide. The procedure used to generate the results in Figures 4.7 and 4.9 includes incubation of MECs with the E-selectin blocking peptide during TNF-α

stimulation and inclusion of the peptide in the erythrocyte suspension perfused over the cells. The inclusion of the peptide in the erythrocyte suspension is necessary to inhibit adhesion. To control for the possibility that the ability of the peptide to inhibit adhesion results from binding of the peptide to the sickle erythrocytes rather than the MECs, sickle erythrocytes are incubated with the peptide for 30 minutes, centrifuged, and resuspended in peptide-free media for perfusion over MECs stimulated with TNF-α for 6 hrs. Results shown in Figure 4.11, Panel A demonstrate that sickle erythrocyte adhesion is not inhibited by this method, suggesting that the peptide is not bound to the erythrocytes at the time of perfusion. The necessity of including the E-selectin blocking peptide in the sickle erythrocyte suspension to inhibit adhesion is possibly due to low affinity of the peptide for E-selectin. That is, the peptide may wash away from E-selectin under the flow conditions of the experiment, necessitating constant replenishment of the peptide to inhibit adhesion.

The E-selectin blocking peptide was developed from a recombinant library and had the highest affinity for E-selectin of all members of the library (Martens et al., 1995). A peptide containing the same amino acids as the E-selectin blocking peptide but with a scrambled sequence was

generated and used like the original peptide to serve as a control. The results shown in Panel B of Figure 4.11 show that the scrambled peptide does not inhibit adhesion of sickle erythrocytes, likely because it does not have affinity for E-selectin.

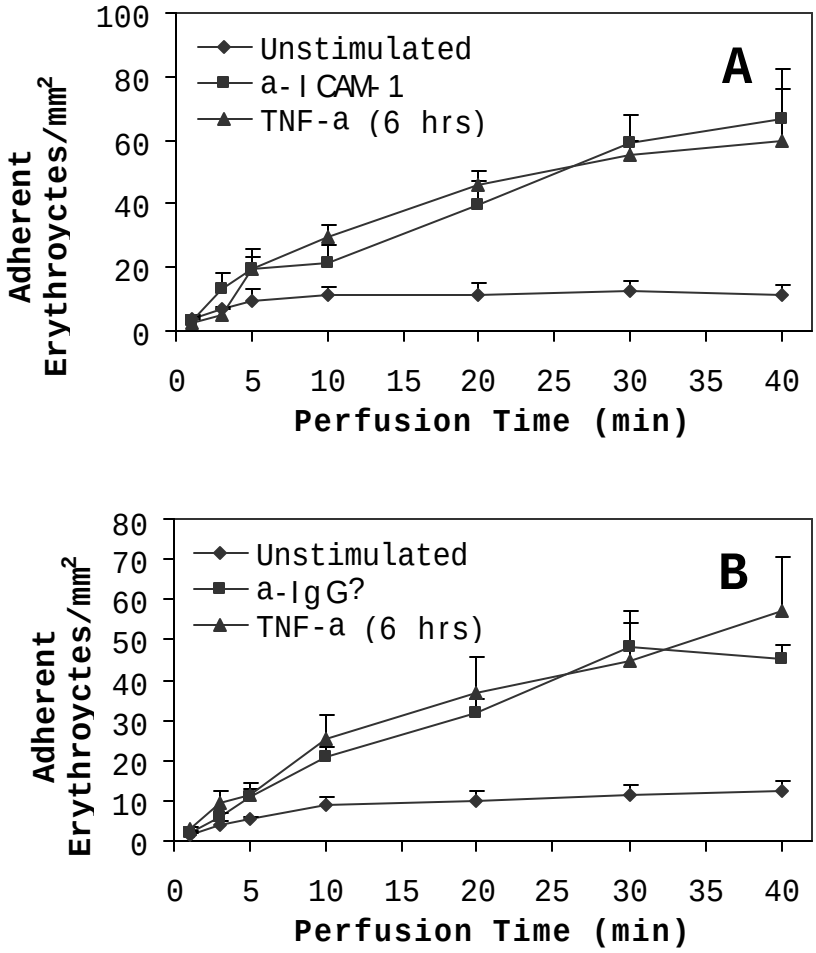


Figure 4.10 Sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs and treated with anti-ICAM-1 or anti-IgGk antibodies. Sickle erythrocyte adhesion to MECs treated with A) anti-ICAM-1 antibody (n=4) or B) anti-IgGκ antibody (n=9). Data for individual blood samples are given in Tables B.15 and B.16.

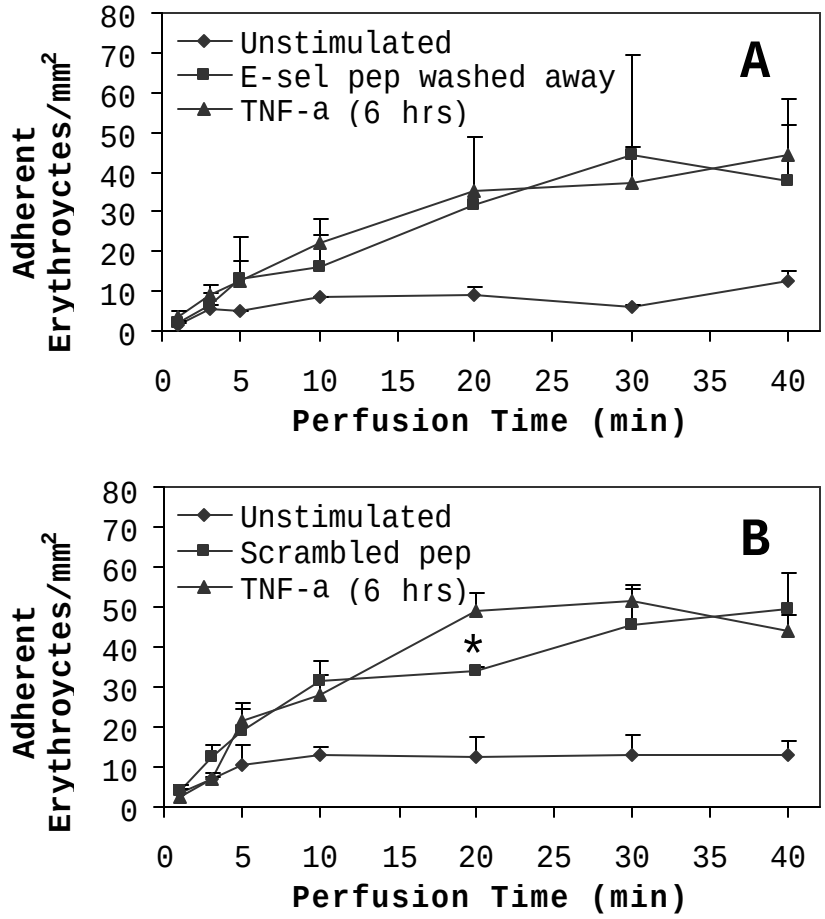


Figure 4.11 Sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs and treated with control peptides. A) Sickle erythrocyte adhesion when erythrocytes were incubated with E-selectin blocking peptide, centrifuged, and resuspended in peptide-free media prior to perfusion (n=3). B) Sickle erythrocyte adhesion when a scrambled version of the E-selectin blocking peptide was used to treat MECs and included in the erythrocyte suspension for flow (n=3). Data for individual blood samples are given in Tables B.17 and B.18.

4.4 Blocking of Sickle Erythrocyte Ligands

As discussed in Section 2.4, adhesion of sickle erythrocytes to vascular endothelium results from binding of ligand molecules on sickle erythrocytes to adhesion molecules or receptors on the endothelial cell surface. The integrin $\alpha_4\beta_1$ has previously been identified as a specific ligand on sickle erythrocytes that binds to endothelial VCAM-1 (Swerlick *et al.*, 1993). A specific erythrocyte ligand for E-selectin has not been identified, but others have suggested that sialic acid on sickle erythrocytes contributes to their adhesion to endothelial cells and another member of the selectin family, P-selectin, under conditions of low (0.1 dyne/cm^2 shear stress) or no flow (Matsui *et al.*, 2001; Montes et al., 2002). However, several studies have also shown that membrane sialic acid content is lower for sickle erythrocytes than for non-sickle erythrocytes (Aminoff *et al.*, 1980; Ekeke & Ibeh, 1988; Westerman *et al.*, 1979).

To determine which erythrocyte ligands support adhesion of sickle erythrocytes to TNF-α stimulated MECs, sickle erythrocytes are incubated with either anti-alpha 4 antibody or neuraminidase, which cleaves sialic acid (Uchida *et al.*, 1979), for 40 minutes at 37°C.

Erythrocytes incubated with neuraminidase are then centrifuged and resuspended in neuraminidase-free media. Treated erythrocytes are perfused over endothelial cells stimulated with TNF-α for 6 hrs. Results are shown in Figure 4.12. Panel A shows that treatment of erythrocytes with anti-alpha 4 antibody does not significantly inhibit adhesion. Panel B shows that treatment with neuraminidase also does not inhibit sickle erythrocyte adhesion.

The inability of these treatments to reduce sickle erythrocyte adhesion is potentially explained by two hypotheses. First, simultaneous inhibition of both the VCAM-1 ligand and the E-selectin ligand on sickle erythrocytes may be necessary for inhibition of erythrocyte adhesion to TNF-α stimulated MECs. Second, sialic acid may not support adhesion of sickle erythrocytes to E-selectin under a physiological shear stress of 1 dyne/cm^2. To test these hypotheses, an E-selectin ligand might be identified by testing treated erythrocyte adhesion to MECs stimulated with TNF-α for only 2 hrs, a stimulation time for which VCAM-1 is not upregulated on MECs. Identification of an E-selectin ligand would allow for simultaneous inhibition of it and erythrocyte $\alpha_4\beta_1$ and testing of the first hypothesis. Alternatively, perfusion experiments in which sickle

erythrocytes treated with anti-α_4 antibody were perfused over MECs treated with anti-E-selectin antibody or peptide and TNF-α could be performed to determine whether mutual inhibition of both adhesion pathways is necessary. Blocking of a single pathway (i.e. use of one antibody or peptide alone) could serve as a control.

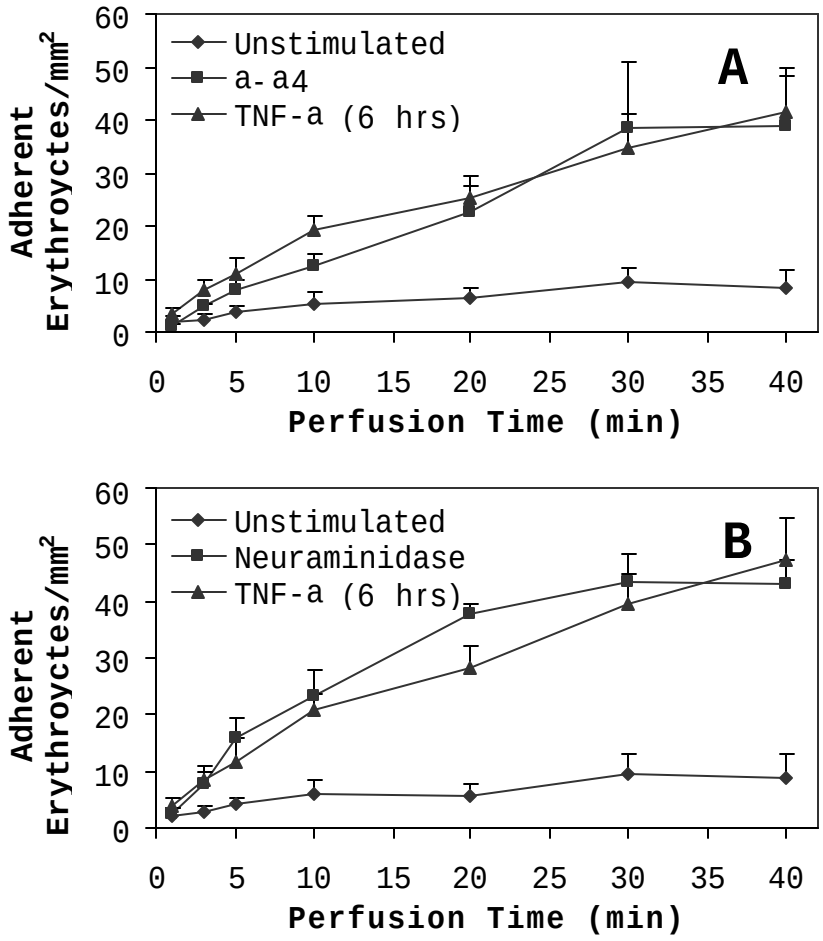


Figure 4.12 Adhesion of sickle erythrocytes treated with anti-alpha 4 antibody or neuraminidase to MECs stimulated with TNF-a for 6 hrs. Sickle erythrocyte adhesion when erythrocytes were treated with A) anti-alpha 4 antibody (n=5) or B) neuraminidase (n=4). Data for individual blood samples are given in Tables B.19 and B.20.

4.5 Chapter Summary

Vascular occlusion by sickle erythrocytes results in many of the clinical manifestations associated with sickle cell anemia. Stimulation of endothelial cells with cytokines such as TNF-α increases adhesion molecule expression and sickle erythrocyte adhesion *in vitro*; however, expression profiles of VCAM-1, E-selectin, and ICAM-1 vary with endothelial phenotype. The data presented in this chapter demonstrate that VCAM-1 and E-selectin support adhesion of sickle erythrocytes, whereas ICAM-1 does not. For microvascular endothelial cells, sickle erythrocyte adhesion to unstimulated endothelium is minimal. Stimulation with TNF-α for 2 hrs increases E-selectin, but not VCAM-1 expression, and increases sickle erythrocyte adhesion by 2.6-fold. Antibody and peptide blocking studies are inconclusive, but suggest that E-selectin may support adhesion of sickle erythrocytes at this stimulation time. Stimulation of MECs with TNF-α for 6 hrs results in significant expression of both VCAM-1 and E-selectin and sickle erythrocyte adhesion is increased 6.3-fold. Antibody and peptide blocking studies show that

both VCAM-1 and E-selectin support adhesion of sickle erythrocytes at this stimulation time.

Together these data show that sickle erythrocyte adhesion is complex *in vitro*. Dominant mechanisms of adhesion vary with cytokine stimulation time, indicating that strategies to inhibit adhesion *in vivo* may need to encompass multiple adhesive mechanisms to be effective. Taking this into account, the following chapters explore inhibition of sickle erythrocyte adhesion by interruption of the common intracellular signaling cascades initiated by cytokines that lead to expression of endothelial adhesion molecules.

CHAPTER 5

INHIBITION OF CYTOKINE INDUCED SICKLE ERYTHROCYTE ADHESION BY ELEVATION OF ENDOTHELIAL INTRACELLULAR cAMP

As discussed previously in Sections 2.4 and 2.5, inhibition of sickle erythrocyte adhesion to vascular endothelial cells is a potential therapeutic strategy for preventing or treating occlusive events in sickle cell anemia, but currently no such therapies exist. One way to inhibit sickle erythrocyte adhesion is to physically block adhesion molecules and ligands supporting adhesion using antibodies or peptides. However, each antibody or peptide is specific for an individual adhesion molecule. The data shown in Chapter 4 and elsewhere in literature (H. A. Brittain et al., 1993; Hebbel, 1997, 2000; Kumar, 1995) suggest that sickle erythrocyte adhesion *in vivo* may be complex, with multiple endothelial adhesion molecules and erythrocyte ligands supporting adhesion simultaneously or at different times during the course of an occlusive event. Thus for physical blocking of sickle erythrocyte adhesion

to be effective, development of a complex cocktail of antibodies and peptides blocking several adhesion molecules and ligands may be necessary.

An alternative strategy to preventing sickle erythrocyte adhesion to endothelial cells is to inhibit the expression of endothelial adhesion molecules by interruption of the intracellular signaling cascades leading to their expression. Many of the known endothelial adhesion molecules supporting sickle erythrocyte adhesion, including VCAM-1 and E-selectin, are upregulated in response to infection and inflammation. Cytokines, including TNF-α, that are released into the blood stream during inflammation initiate cell signaling resulting in adhesion molecule expression. Several studies have demonstrated that the ubiquitous intracellular signaling molecule cAMP suppresses inflammatory response (Galea & Feinstein, 1999; Otsuki et al., 2001; Pober et al., 1993) by controlling endothelial cell signaling. The mechanisms by which these effects occur are outlined in more detail in Section 2.6. The studies presented in this chapter explore how elevated endothelial cAMP affects TNF-α induced VCAM-1, E-selectin, and ICAM-1 expression on MECs, and how regulation of these adhesion molecules affects sickle erythrocyte adhesion.

5.1 The Effect of Reagents that Increase Intracellular cAMP on TNF-a Induced Adhesion Molecule Expression

Elevated endothelial intracellular cAMP is thought to suppress inflammatory response, particularly cytokine-induced adhesion molecule expression. *In vitro*, cAMP levels can be elevated primarily by 1) activating adenylate cyclase with forskolin (Fsk) to produce more cAMP, 2) inhibiting the phosphodiesterases that break down cAMP with various reagents, including the methyl xanthine derivative isobutylmethylxanthine (IBMX), and 3) applying cell-permeable cAMP analogues such as dibutyryl cAMP (Bt_2cAMP). The effects of increasing cAMP by each of these methods on adhesion molecule expression are shown in the following section.

5.1.1 The Effect of Forskolin on VCAM-1, E-selectin, and ICAM-1 Expression

Figure 5.1 shows the effects of forskolin on VCAM-1, E-selectin, and ICAM-1 expression on MECs. The first bar in each panel shows expression on unstimulated MECs, and the second bar shows increased expression due to 6 hrs of TNF-α stimulation. The remaining bars show expression when MECs are pretreated with the given concentration of

forskolin for 30 minutes prior to TNF-α stimulation. Panel A shows that VCAM-1 expression is reduced in a concentration dependent manner due to forskolin pretreatment. The reduction becomes statistically significant, but does not return to baseline, with 1000 μM forskolin. Results follow a similar trend for E-selectin (Panel B). Forskolin pretreatment at concentrations as low as 10 μM significantly inhibits E-selectin expression, with greater inhibition occurring at higher concentrations. Panel C shows that ICAM-1 expression is not significantly altered by pretreatment with forskolin.

In addition to the concentration of forskolin used, another important parameter to consider is the time of application. Figure 5.2 shows the effect of forskolin pretreatment times ranging from 0 to 120 minutes on VCAM-1, E-selectin, and ICAM-1 expression induced by TNF-α stimulation for 6 hrs. In addition, the last two bars on each panel show expression when MECs are pretreated with forskolin for either 30 or 120 minutes, and forskolin is also applied continuously through the duration of TNF-α stimulation. A forskolin concentration of 100 μM was used for experiments shown in Figure 5.2. Panel A indicates that TNF-α induced VCAM-1 expression is significantly

inhibited by as little as 15 minutes of pretreatment with forskolin. Pretreatment for longer times does not further repress VCAM-1 expression. However, pretreatment and continuous presence of forskolin during TNF-α stimulation reduces VCAM-1 levels to baseline (levels on unstimulated MECs). Panel B of Figure 5.2 shows a similar trend for E-selectin.

Pretreatment with forskolin significantly inhibits E-selectin expression, but greater inhibition is achieved when forskolin is also present during TNF-α stimulation. Even continous presence of forskolin, however, is not sufficient to completely inhibit E-selectin expression. Again, Panel C shows that ICAM-1 expression is not significantly inhibited by treatment of MECs with forskolin.

Together these data demonstrate that forskolin inhibits VCAM-1 and E-selectin, but not ICAM-1 expression on TNF-α stimulated MECs. Forskolin is most effective when it is applied both prior to and during TNF-α stimulation, suggesting that treatment time is more important than treatment concentration. Possible explanations for the need for continuous forskolin presence are discussed in Section 5.2.2.

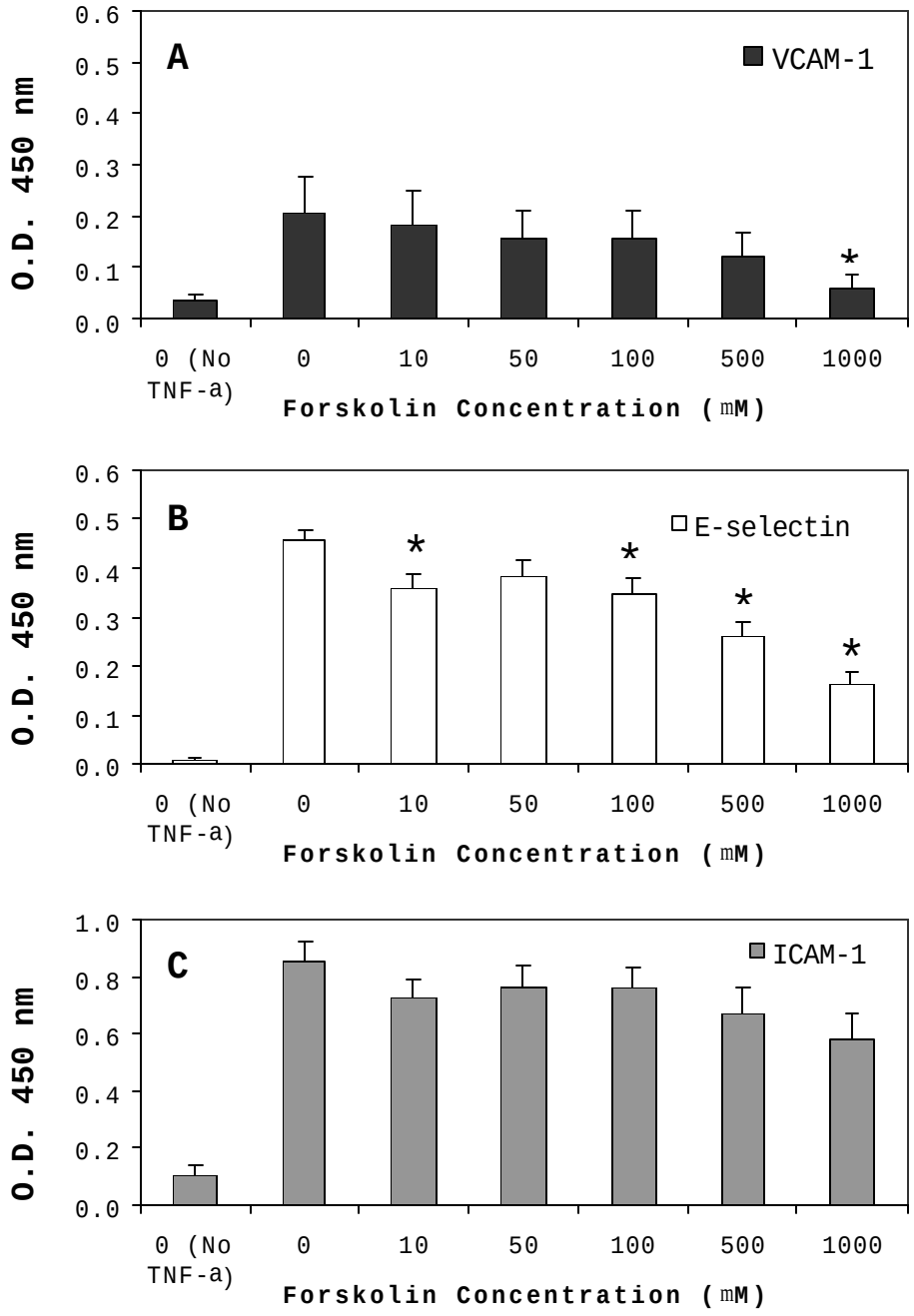


Figure 5.1 Effect of forskolin pretreatment concentration on TNF-a induced adhesion molecule expression. Surface expression of A) VCAM-1, B) E-selectin, and C) ICAM-1 on MECs treated with forskolin for 30 min then TNF-α for 6 hrs (n=6). *Indicates statistically significant difference from MECs treated with only TNF-α (0 μM forskolin). Data for individual experiments are given in Table B.21.

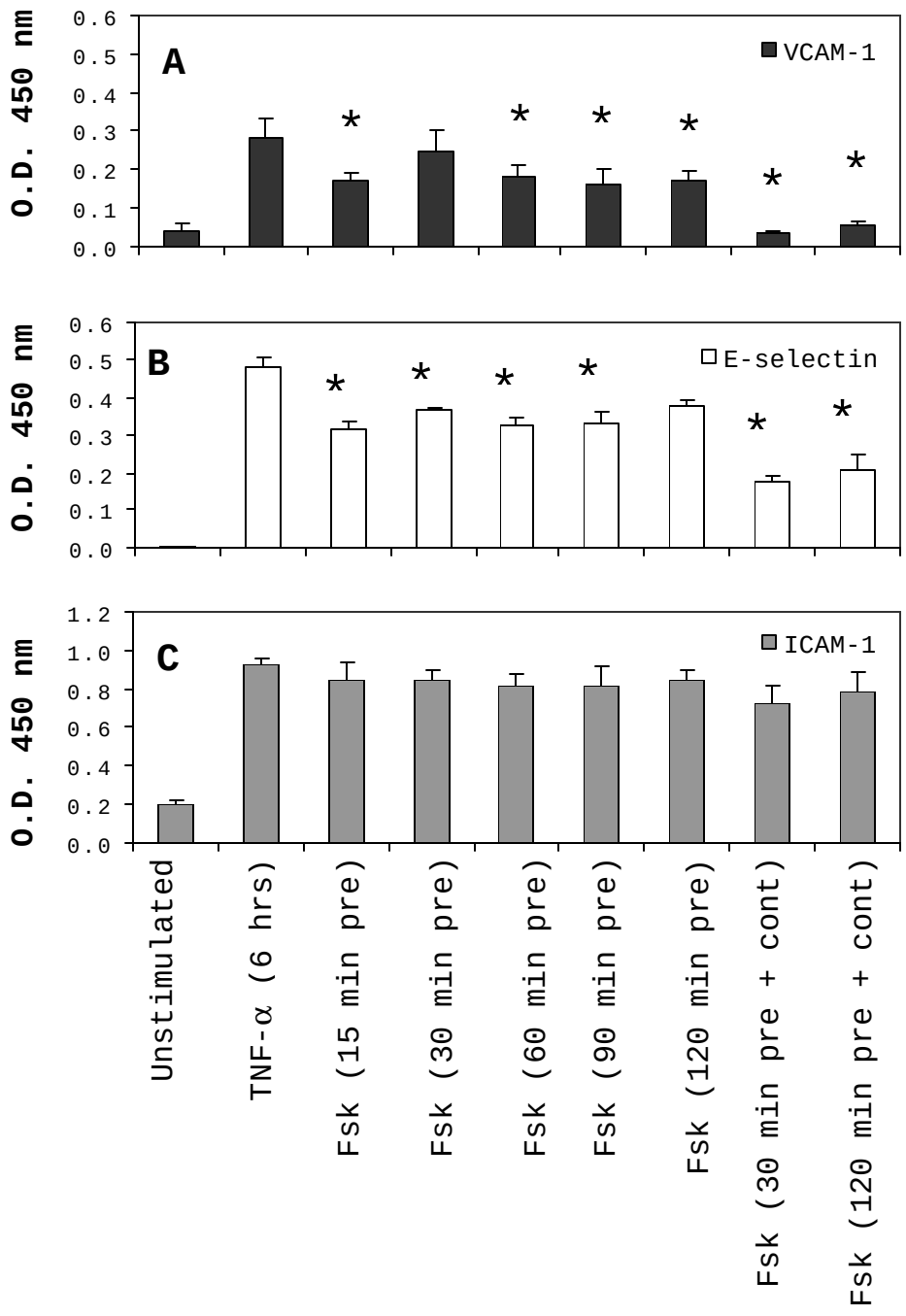


Figure 5.2 Effect of forskolin pretreatment time on TNF-α induced adhesion molecule expression. Surface expression of A) VCAM-1, B) E-selectin, and C) ICAM-1 on MECs pretreated with 100 μM forskolin for the indicated time then TNF-α and/or forskolin for 6 hrs (n=4). *Indicates statistically significant difference from MECs treated with only TNF-α. Data for individual experiments are given in Table B.22.

5.1.2 The Effect of IBMX on VCAM-1, E-selectin, and ICAM-1 Expression

The ability of forskolin to regulate adhesion molecule expression is likely due to elevation of intracellular cAMP levels. To further demonstrate the role of cAMP in regulating adhesion molecule expression, MECs are treated with IBMX, which elevates cAMP by a different mechanism (inhibition of phosphodiesterases) than forskolin, for 30 minutes prior to and during 6 hrs of TNF-α stimulation. Results are shown in Figure 5.3.

Panel A of Figure 5.3 shows that pretreatment and continuous presence of IBMX at concentrations of 50 μM or greater significantly inhibits TNF-α induced VCAM-1 expression, and degree of inhibition appears concentration dependent. Panel B shows that E-selectin expression is not significantly inhibited at concentrations less than 1000 μM, and ICAM-1 expression is not significantly affected by IBMX.

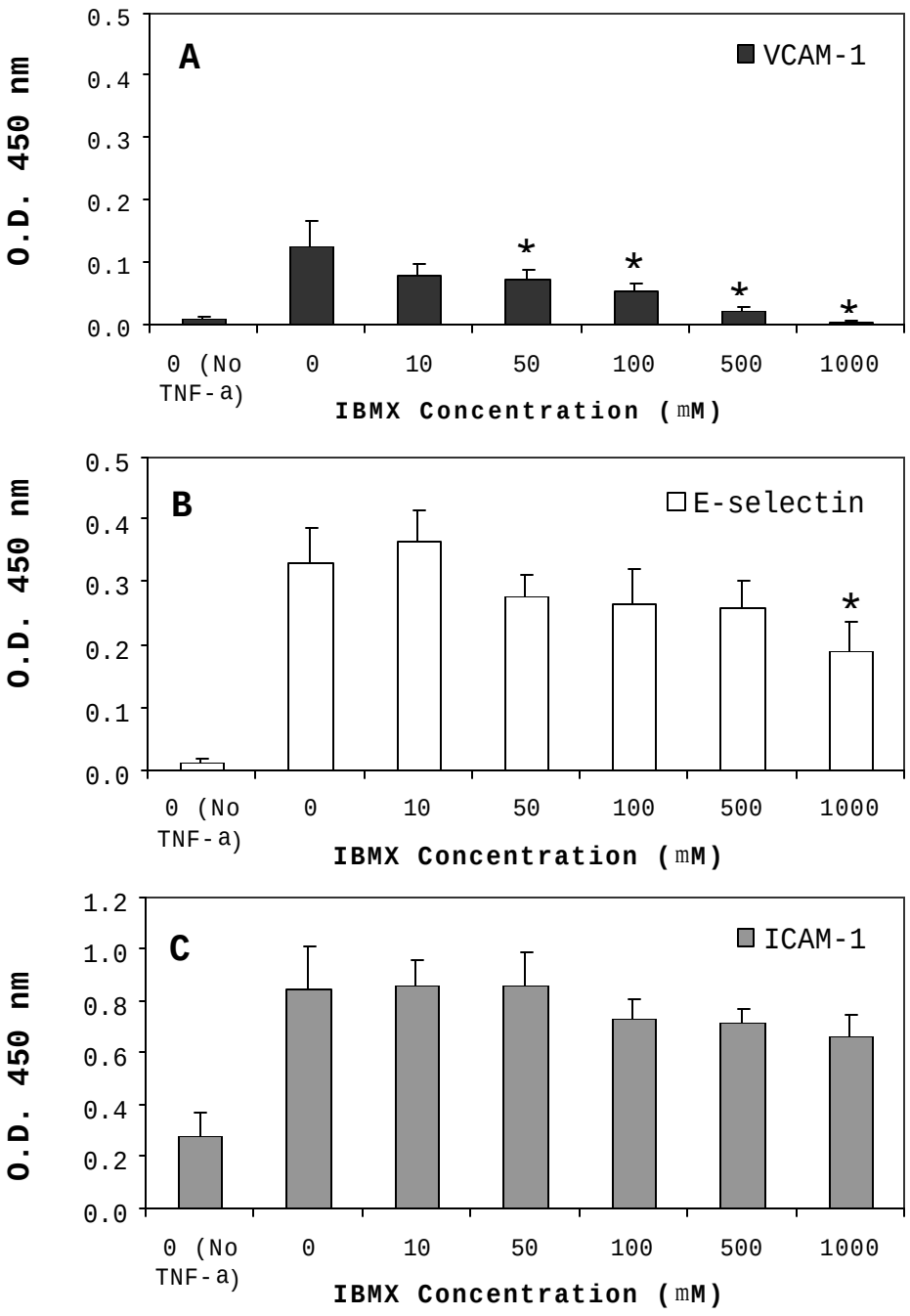


Figure 5.3 Effect of IBMX concentration on TNF-a induced adhesion molecule expression. Surface expression of A) VCAM-1, B) E-selectin, and C) ICAM-1 on MECs pretreated with IBMX for 30 min then TNF-α and IBMX for 6 hrs (n=5). *Indicates statistically significant difference from MECs treated with only TNF-α (0 μM IBMX). Data for individual experiments are given in Table B.23.

5.1.3 The Effect of Bt_2cAMP on VCAM-1, E-selectin, and ICAM-1 Expression

In addition to regulation of the enzymes that make and break down cAMP in endothelial cells, another method to elevate intracellular cAMP is to supply cell-permeable cAMP analogues in the cell culture media, which diffuse into the cells and produce physiological responses like cAMP. The effect of pretreatment and continuous presence of one cAMP analogue, Bt_2cAMP, at varying concentrations on TNF-α induced VCAM-1, E-selectin, and ICAM-1 expression is shown in Figure 5.4 below. Panel A shows that Bt_2cAMP inhibits VCAM-1 expression in a concentration dependent manner, with inhibition becoming statistically significant at Bt_2cAMP concentrations of 100 μM or higher. E-selectin expression is also inhibited by Bt_2cAMP (Panel B), while ICAM-1 is not except at a concentration of 1000 μM (Panel C).

Pretreatment and continuous presence of either Fsk, IBMX, or Bt_2cAMP during TNF-α stimulation inhibits, but does not completely abolish, VCAM-1 and E-selectin expression. Literature suggests that treatment of endothelial cells with both Fsk and cilostazol, a phosphodiesterase inhibitor like IBMX, results in greater inhibition of VCAM-1 expression than treatment with either reagent alone (Otsuki et al., 2001). However, a study of the effects of multiple

cAMP-elevating reagents used simultaneously on TNF-α induced adhesion molecule expression is not undertaken in this thesis.

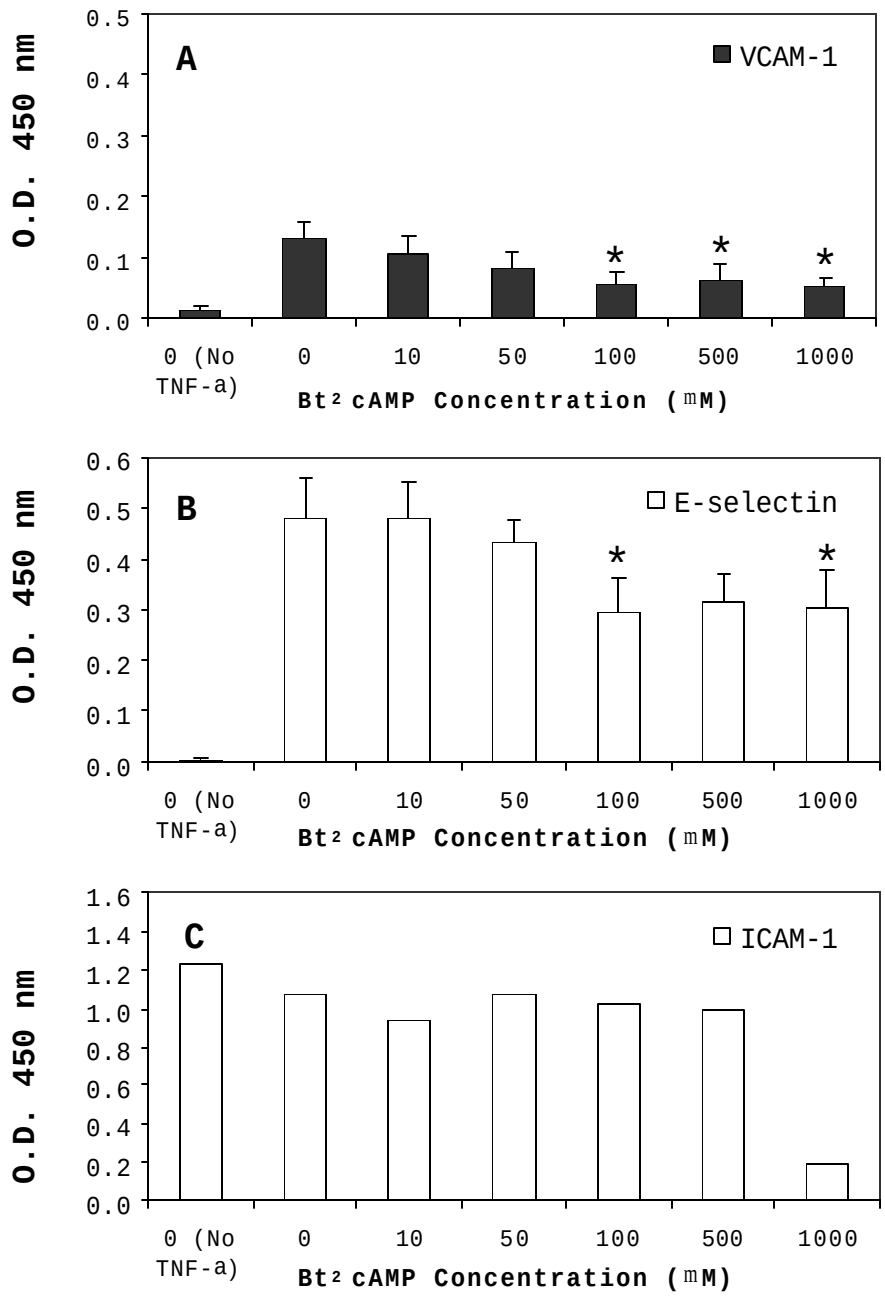


Figure 5.4 Effect of Bt_2cAMP concentration on TNF-α induced adhesion molecule expression. Surface expression of A) VCAM-1 (n=5), B) E-selectin (n=5), and C) ICAM-1 (n=1) on MECs pretreated with Bt_2cAMP for 30 min then TNF-α and Bt_2cAMP for 6 hrs. *Indicates statistically significant difference from MECs treated with only TNF-α (0 μM Bt_2cAMP). Data from individual experiments are presented in Table B.24.

5.1.4 Comparison of Forskolin Effect on MECs stimulated with TNF-α for 2 or 6 hrs

A summary of the effects of forskolin pretreatment and continuous presence during 6 hrs of TNF-α stimulation is presented in Figure 5.5, Panel A (the full study is shown in Figure 5.2). In Figure 5.5 and all following figures, the term “pretreatment,” abbreviated “pre”, refers to a 30 minute treatment with the specified compound prior to TNF-α stimulation. The term “continuous”, abbreviated “cont,” refers to treatment with the specified compound during TNF-α stimulation. Figure 5.5, Panel A reiterates that for 6 hrs of TNF-α stimulation, VCAM-1 and E-selectin expression are upregulated. Pretreatment with forskolin inhibits E-selectin, but not VCAM-1 expression. Pretreatment and continuous presence of forskolin during TNF-α stimulation inhibits VCAM-1 and E-selectin expression, but not ICAM-1.

Similar experiments were conducted with MECs stimulated with TNF-α for 2 hrs, and the results are shown in Figure 5.5, Panel B. The data demonstrate that VCAM-1 is not upregulated on MECs after 2 hrs of stimulation and is not affected by forskolin. E-selectin is upregulated, and expression is inhibited by pretreatment with forskolin

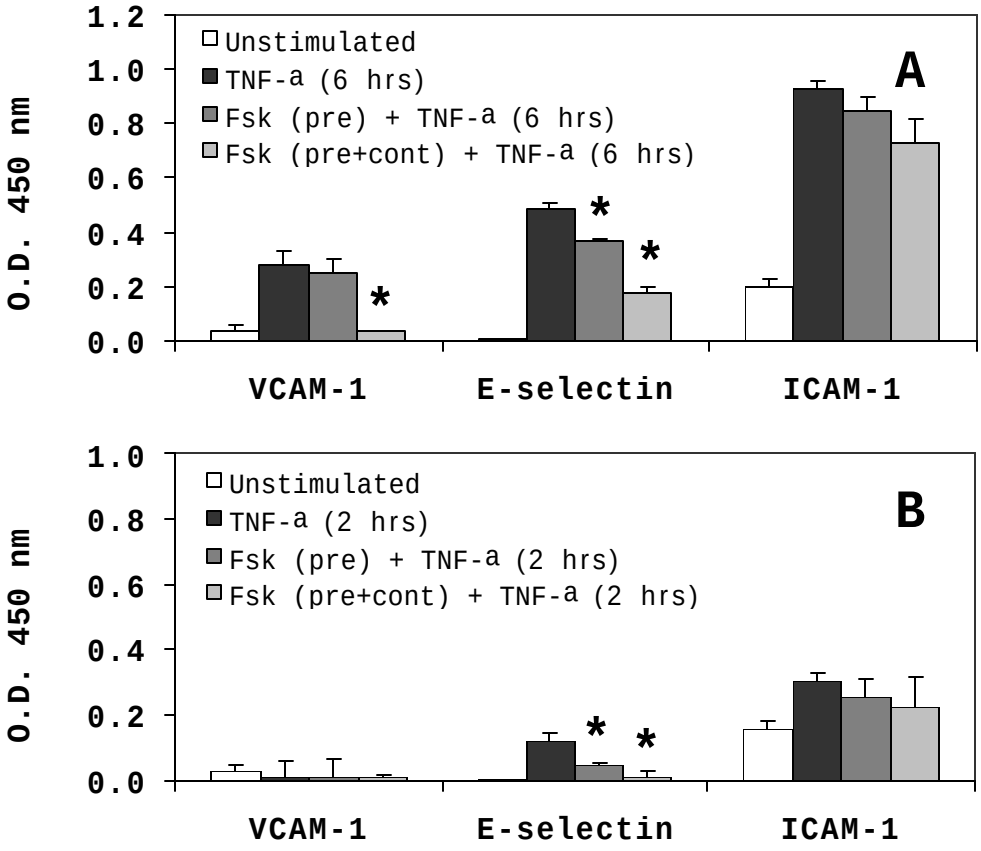


Figure 5.5 Comparison of the effect of forskolin on MECs stimulated with TNF-a for 2 or 6 hrs. Surface expression of VCAM-1, E-selectin, and ICAM-1 on MECs pretreated with Fsk for 30 min then TNF-α alone or TNF-α and Fsk for 2 (n=6) or 6 (n=4) hrs. *Indicates statistically significant difference from MECs treated with only TNF-α. Data are also presented in Table B.25.

and is further inhibited by pretreatment and continuous presence of the cAMP-elevating reagent.

5.1.5 Effect of Fsk, IBMX, and Bt_2cAMP alone on VCAM-1, E-selectin, and ICAM-1 expression

To test for the possibility that increasing cAMP might alter adhesion molecule expression on MECs by means other than interruption of cytokine induced signaling cascades, MECs were treated with Forskolin, IBMX, or Bt_2cAMP alone for 6.5 hours and VCAM-1, E-selectin, and ICAM-1 expression were measured. The results shown in Table 5.1 demonstrate that treatment with these reagents alone does not induce adhesion molecule expression, since optical density does not change with treatment.

Additionally, to ensure that the ability of cAMP elevating reagents to inhibit adhesion molecule expression was not due to cell damage or death, endothelial monolayers treated or not with reagents that increase cAMP were photographed under light microscopy. Also, a live/dead assay was performed as described in Section 3.6, and photographs of the cells were taken using confocal microscopy. Live cells are stained green and dead cells are stained red. Pictures are shown in Appendix C and

reveal that viability was not affected by endothelial treatment with reagents that increase cAMP.

Table 5.1 Effect of Fsk, IBMX, or Bt_2cAMP alone on adhesion molecule expression. Surface expression of VCAM-1, E-selectin, and ICAM-1 on MECs treated with 100 μM Fsk, 500 μM IBMX, or 500 μM Bt_2cAMP for 6.5 hrs (n=2). Data for individual experiments are presented in Table B.26.

Endothelial Treatment	VCAM-1	E-selectin	ICAM-1
Unstimulated	0.019	0.033	0.134
Fsk	0.018	0.027	0.129
IBMX	0.032	0.035	0.104
Bt_2cAMP	0.026	0.069	0.127

5.2 The Effect of Reagents that Increase Endothelial Intracellular cAMP on TNF-α Induced Sickle Erythrocyte Adhesion

The data presented in Section 5.1 demonstrate that interruption of cytokine induced cell signaling cascades is achieved by elevation of endothelial intracellular cAMP. Such interruption inhibits TNF-α induced VCAM-1 and E-selectin expression, but not ICAM-1 expression. Data presented in Section 5.2 translate from the biochemical ELISA assay to the functional adhesion assay. That is, in

this section, the effects of increased endothelial cAMP on TNF-α induced sickle erythrocyte adhesion are examined.

5.2.1 Inhibition of Sickle Erythrocyte Adhesion to MECs stimulated with TNF-a for 2 hrs

Figure 5.6 demonstrates the effects of 30 minute pretreatment with forskolin on sickle erythrocyte adhesion induced by 2 hrs of TNF-α stimulation. Pretreatment with forskolin inhibits sickle erythrocyte adhesion by 74% after 20 minutes of erythrocyte perfusion, 63% after 30 minutes, and 48% after 40 minutes. Inhibition is statistically significant at 20 and 30 minutes of perfusion. That forskolin pretreatment inhibits, but does not completely abolish sickle erythrocyte adhesion is potentially explained by comparison of Figure 5.6 with Figure 5.5, Panel B. Pretreatment alone does not completely inhibit E-selectin expression, thus it is likely that residual E-selectin contributes to sickle erythrocyte adhesion after forskolin pretreatment. In addition, it is possible that blood age (that is, the amount of time that passes between when the blood is drawn from the patient and when it is used for flow experiments) contributes to incomplete inhibition. Blood samples were over 16 hrs old when used

in the flow adhesion assays presented in this section. A more detailed analysis of the effect of blood age on adhesion is given in Section 5.4.

5.2.2 Inhibition of Sickle Erythrocyte Adhesion to MECs stimulated with TNF-a for 6 hrs

The data presented in Figure 5.7, Panel A demonstrate the effects of forskolin pretreatment and/or continuous presence when MECs are stimulated with TNF-α for 6 hrs. Unlike the data shown in Figure 5.6 for 2 hrs of TNF-α stimulation, pretreatment alone of MECs with forskolin prior to 6 hrs of TNF-α stimulation does not significantly inhibit sickle erythrocyte adhesion. However, when forskolin is present both prior to and during TNF-α stimulation, adhesion is inhibited by 59% after 40 minutes of perfusion. Panels B and C of Figure 5.7 show similar results with pretreatment and continuous presence of IBMX or Bt_2cAMP, with IBMX inhibiting adhesion by 64% and Bt_2cAMP by 64%. Together these data show that sickle erythrocyte adhesion to TNF-α stimulated MECs is inhibited by elevation of intracellular cAMP.

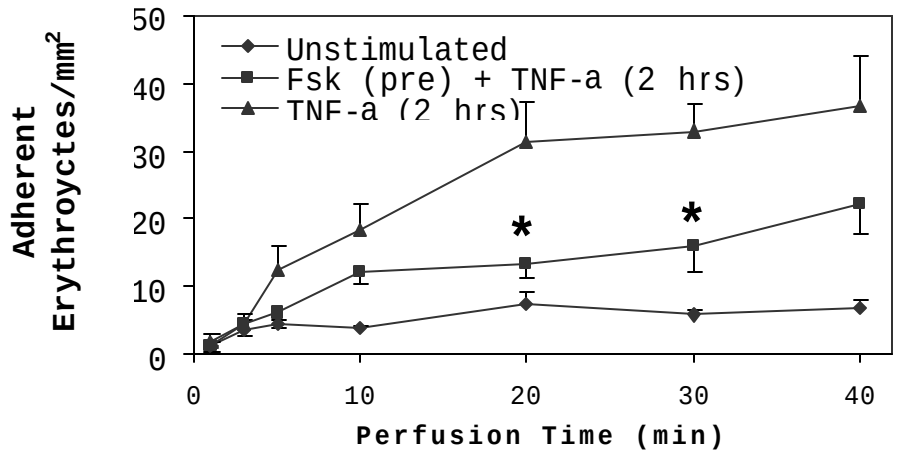


Figure 5.6 Effect of 100 µM forskolin pretreatment on sickle erythrocyte adhesion induced by 2 hrs TNF-a stimulation. *Indicates statistically significant difference from MECs treated with only TNF-α (n=5). Data for individual blood samples are presented in Table B.27.

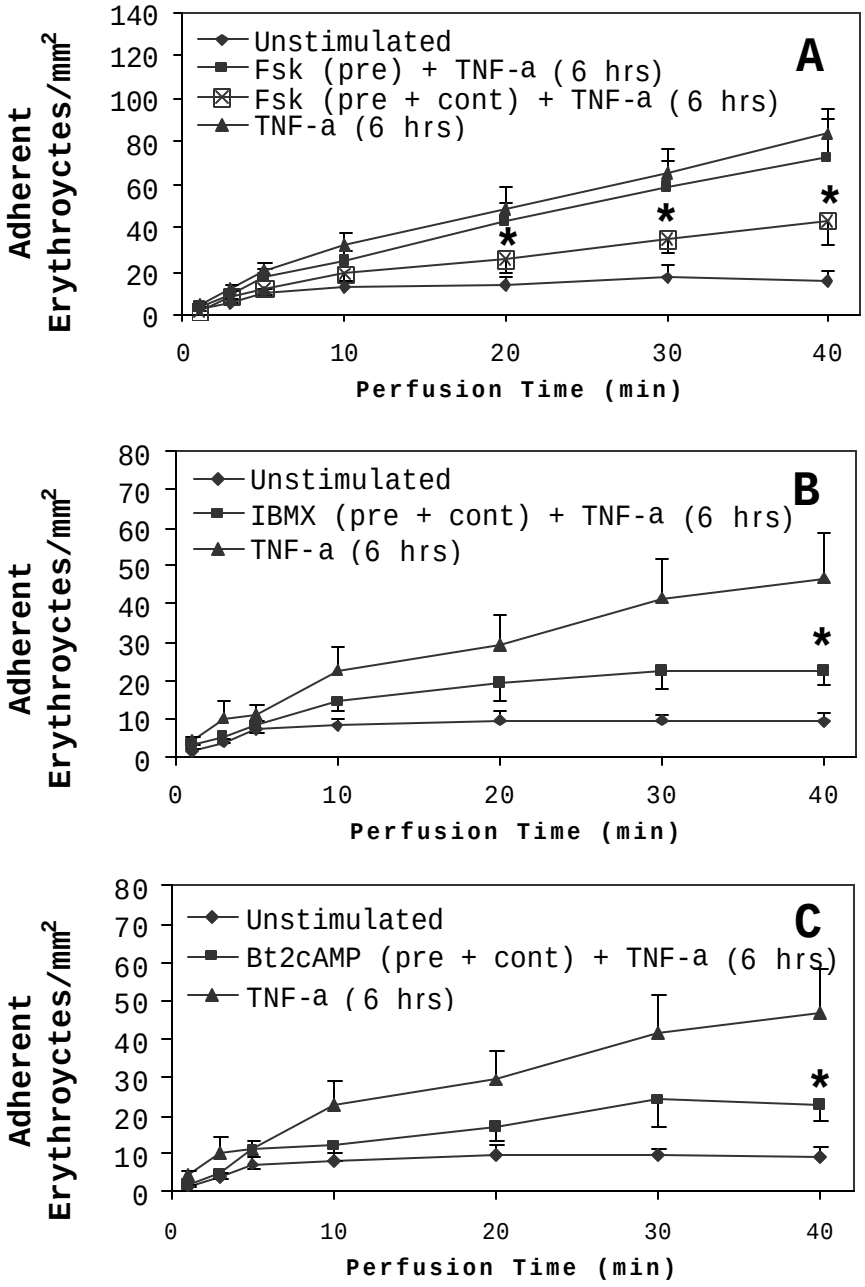


Figure 5.7 Effect of reagents that elevate endothelial cAMP on sickle erythrocyte adhesion induced by 6 hrs TNF-a stimulation. Pretreatment and/or continuous presence of A) 100 μM Forskolin (n=10), B) 500 μM IBMX (n=8), or C) 500 μM Bt_2cAMP (n=8) inhibits sickle erythrocyte adhesion. *Indicates statistically significant difference from MECs treated with only TNF-α. Data for individual blood samples are presented in Tables B.28-9.

The need for continuous presence of forskolin during 6 hrs of TNF-α stimulation may be explained by the data presented in Figures 5.8 and 5.9. Figure 5.8 shows endothelial cAMP concentrations when MECs are pretreated with 100 μM forskolin for 30 minutes, then stimulated with TNF-α alone for 6 hrs. The first bar in Figure 5.8 shows cAMP levels in quiescent, untreated MECs. The second bar shows that treatment of MECs with 100 μM forskolin for 30 minutes induces a 4-fold increase in cAMP concentration. However, the remaining bars show that when forskolin is removed and MECs are then stimulated with TNF-α alone for 6 hrs, cAMP levels are significantly diminished within 0.5 hrs of forskolin removal and return to baseline within 2 hrs. On the other hand, Figure 5.9 shows that when MECs are pretreated with forskolin and it is also present during TNF-α stimulation, cAMP levels attenuate much more slowly, reaching a trough at 2 hrs of stimulation and then climbing again at 4 and 6 hrs. Concentrations never reach those of untreated MECs. Thus one reason that forskolin may need to be continuously present to inhibit adhesion molecule expression and sickle erythrocyte adhesion long-term is that cAMP levels must be elevated through the duration of cytokine stimulation to inhibit transcription of adhesion

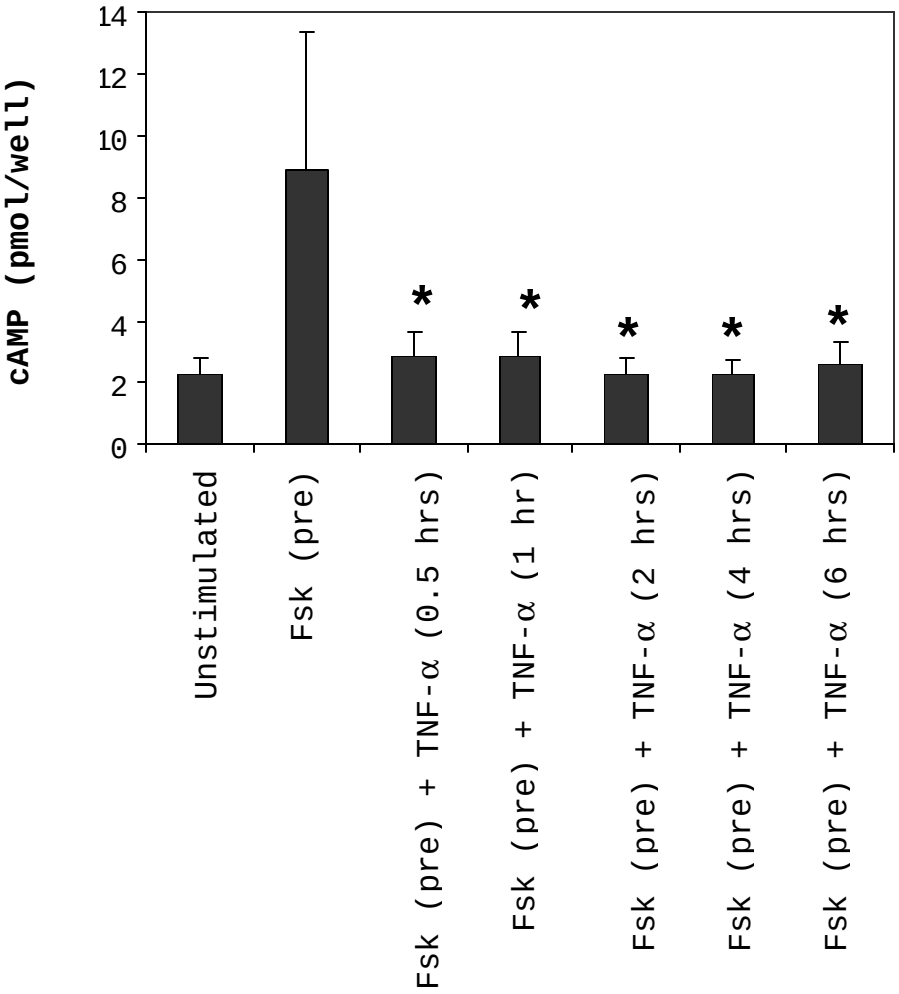


Figure 5.8 Effect of forskolin pretreatment on endothelial cAMP concentration over 6 hrs of TNF-a stimulation. *Indicates statistically significant difference from cAMP concentration just after pretreatment (n=3). Data for individual experiments are presented in Table B.30.

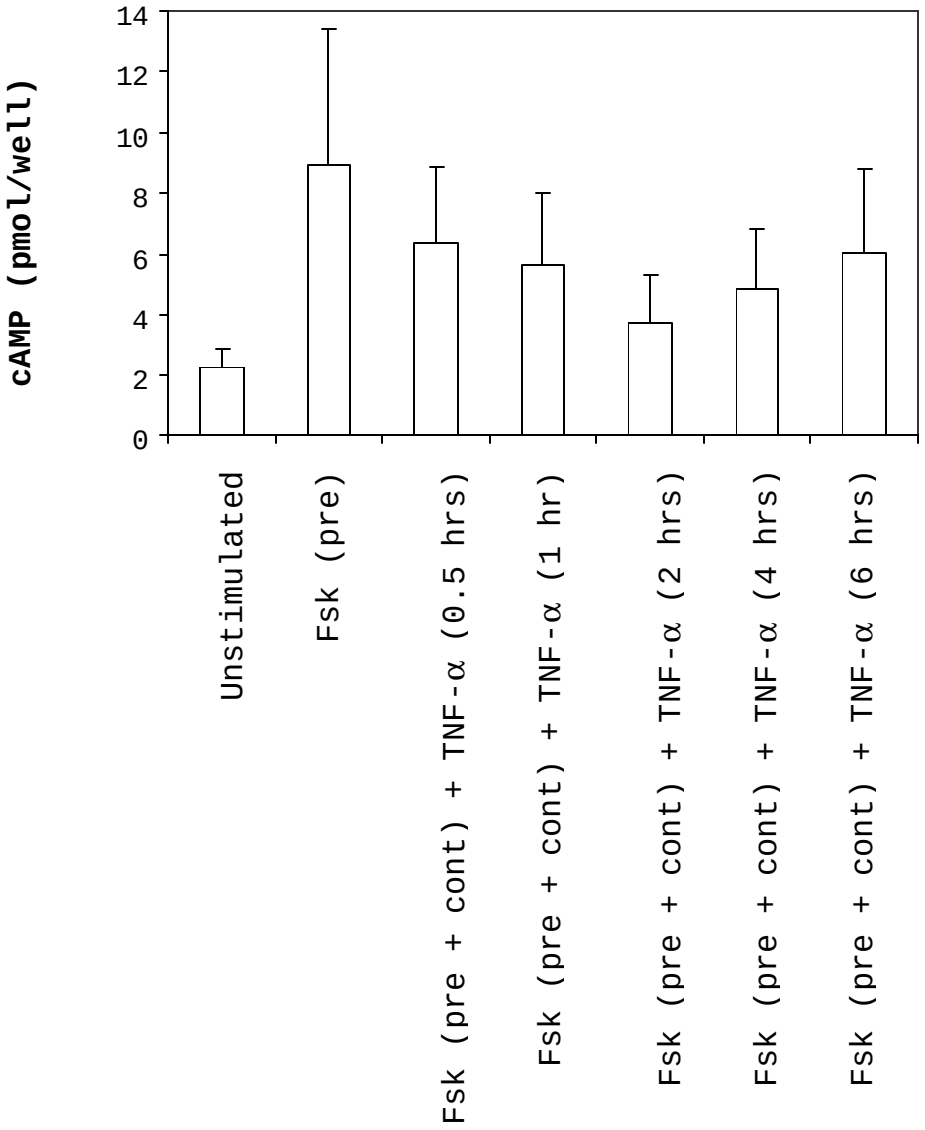


Figure 5.9 Effect of forskolin pretreatment and continous presence on endothelial cAMP concentration over 6 hrs of TNF-a stimulation. *Indicates statistically significant difference from cAMP concentration just after pretreatment (n=3). Data for individual experiments are presented in Table B.30.

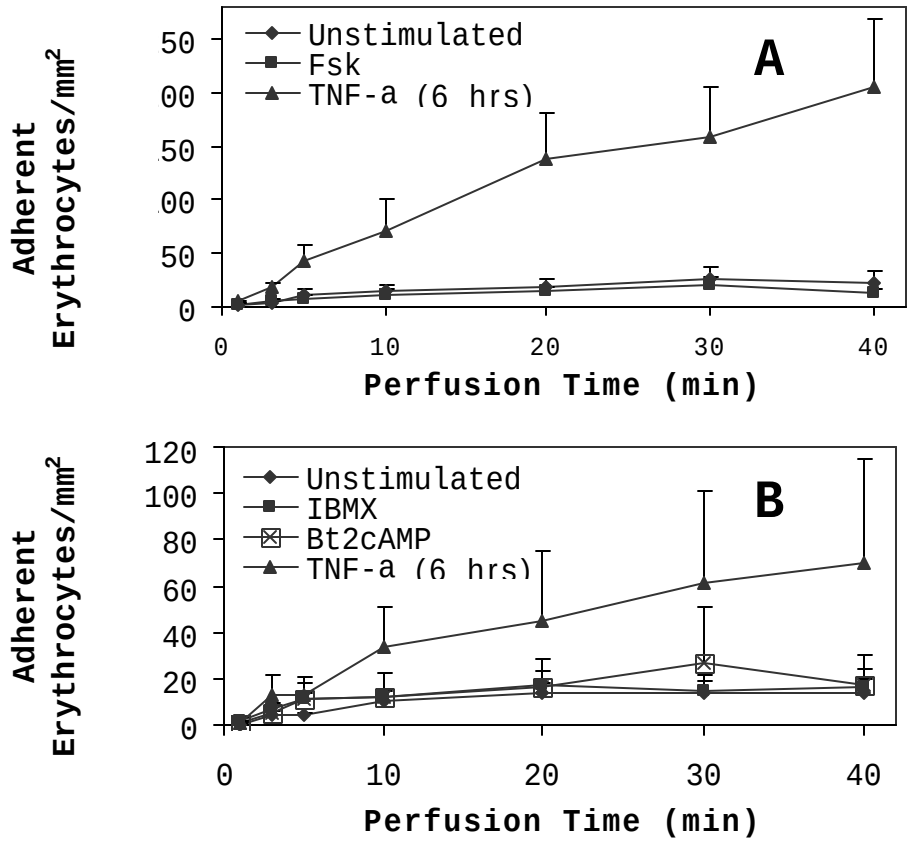


Figure 5.10 Effect of reagents that increase endothelial intracellular cAMP on sickle erythrocyte adhesion. A) 100 μM forskolin (n=4), B) 500 μM IBMX, and 500 μM Bt_2cAMP (n=3) alone (without TNF-α) do not affect sickle erythrocyte adhesion to MECs. Data for individual blood samples are presented in Tables B.31 and B.32.

molecules. A one-time spike in cAMP concentration may delay expression of VCAM-1 and E-selectin, but this delay is less noticeable after 6 hrs of TNF-α stimulation than after 2 hrs. Also, it is interesting to note that despite continuous presence of forskolin, Figure 5.9 shows that cAMP levels are highest after 30 minutes of forskolin treatment and decline thereafter. This may be due to activation of phosphodiesterases, which break down cyclic AMP to AMP. It is not clear from these experiments how high cAMP levels must be to sustain suppression of adhesion molecule expression in the presence of cytokines or if adhesion molecule suppression is sustainable over longer cytokine stimulation times. More detailed experiments need be undertaken to determine kinetics of suppression (See Section 8.1).

Also important to note is that despite continuous presence of cAMP-elevating reagents, complete inhibition of sickle erythrocyte adhesion is not achieved (Figure 5.7). The inability these reagents to completely inhibit adhesion of sickle erythrocytes is potentially explained by three theories. First, while increasing cAMP inhibits TNF-α induced VCAM-1 and E-selectin expression, it could potentially upregulate expression of unknown receptors that promote adhesion. This scenario is unlikely, however,

since Figure 5.10 demonstrates that treatment of MECs with Forskolin, IBMX, or Bt_2cAMP alone for 30 min or 6.5 hrs does not increase sickle erythrocyte adhesion. Another possibility is that the age of the blood reduces the percent inhibition achieved by cAMP elevating compounds. The effect of blood age on adhesion is discussed in Section 5.4.

A likely theory is that incomplete inhibition of sickle erythrocyte adhesion is due to incomplete inhibition of VCAM-1 and E-selectin expression. This theory could be tested in experiments in which endothelial cells are treated with cAMP-elevating reagents and monoclonal antibodies to block residual adhesion molecules during TNF-α stimulation. ELISA data (Figures 5.2-5.4) demonstrate that treatment of MECs with any of the cAMP-inducing reagents does not completely abolish receptor expression. One potential explanation for incomplete inhibition of receptor expression is that others have shown that chronic exposure to cAMP (> 6-8 hrs) may result in a refractory period in which cells are no longer responsive to cAMP stimulation (Krall & Jamgotchian, 1987; Montminy, 1997). Regardless, the data suggest presence of a relatively small number of VCAM-1 or E-selectin molecules may be sufficient to support significant erythrocyte adhesion, indicating

that greater understanding of the biophysics of sickle cell adhesion to VCAM-1 and E-selectin is important to its prevention.

5.3 The Effect of Reagents that Increase Intracellular cAMP in Sickle Erythrocytes on Their Adhesion to MECs

Since *in vivo* modulation of endothelial intracellular cAMP would likely require concomitant modulation of sickle erythrocyte cAMP concentrations, it is important to understand the effects of cAMP-elevating agents on sickle erythrocytes and their adhesion to MECs. Others have shown that treatment of sickle erythrocytes, rather than the endothelium, with forskolin can increase adhesion to endothelial $\alpha_5\beta_3$ by activation of ICAM-4 on sickle erythrocytes (Zennadi *et al.*, 2004). Those results were obtained by statically incubating erythrocytes with endothelial cells, then rinsing at 1 dyne/cm^2 shear stress. The results presented in the following section demonstrate the effects of treatment of sickle erythrocyte with reagents that increase cAMP on adhesion to MECs under conditions of continuous flow (without static incubation).

5.3.1 Adhesion of SSRBCs Treated with cAMP Elevating Reagents to Unstimulated MECs

Figure 5.11 shows the effects of sickle erythrocyte treatment with reagents that increase cAMP on adhesion to unstimulated MECs. Because some blood samples respond to treatment with these reagents and others do not, results are shown for individual blood samples, rather than the average of all samples. Data shown are for 40 minutes of erythrocyte perfusion. A complete table of all data for each blood sample (that is, numbers of adherent erythrocytes at all perfusion time points) as well as the averages is given in Appendix B, Tables B.33 and B.34. Also, because blood age might affect the ability of erythrocytes to respond to treatment with cAMP elevating reagents, only blood samples for which experiments were completed within 16 hrs of blood drawing are shown in Figure 5.11. The effect of blood age on sickle erythrocyte adhesion is discussed in Section 5.4.

Panel A of Figure 5.11 shows the effect of pretreatment of sickle erythrocytes for 40 minutes with 100 μM forskolin prior to perfusion on their adhesion to unstimulated MECs. For the four blood samples tested, two respond to forskolin pretreatment, resulting in increased adhesion to MECs. Similarly, panel B shows that

pretreatment of sickle erythrocytes with IBMX results in increased adhesion for 2 out of 3 blood samples, whereas Panel C shows increased adhesion due to erythrocyte pretreatment with Bt_2cAMP in 1 out of 3 blood samples.

These results are partially consistent with those obtained by Zennadi, et al. While their data do not show non-responsive patient samples, it is important to note that the flow conditions of the two experiments were not identical. Rather, Zennadi, et al. followed a pattern of static attachment followed by detachment under flow, while the data presented in Figure 5.11 were gathered under conditions of continuous flow for attachment. As established in Section 2.4.3, low or no flow conditions tend to promote higher numbers of adherent sickle erythrocytes via lower-affinity binding mechanisms; thus by corollary it is reasonable to believe that the flow regime established by Zennadi, et al. would result in more sickle erythrocyte adhesion in more patients than the flow regime established in this work. Regardless, both the data presented in Figure 5.11 and by Zennadi, et al. suggest that increased cAMP in sickle erythrocytes may promote their adhesion to unstimulated MECs in some patients. Thus more experiments are necessary to more clearly define flow

conditions and red cell activation states under which this binding might be important to patient health.

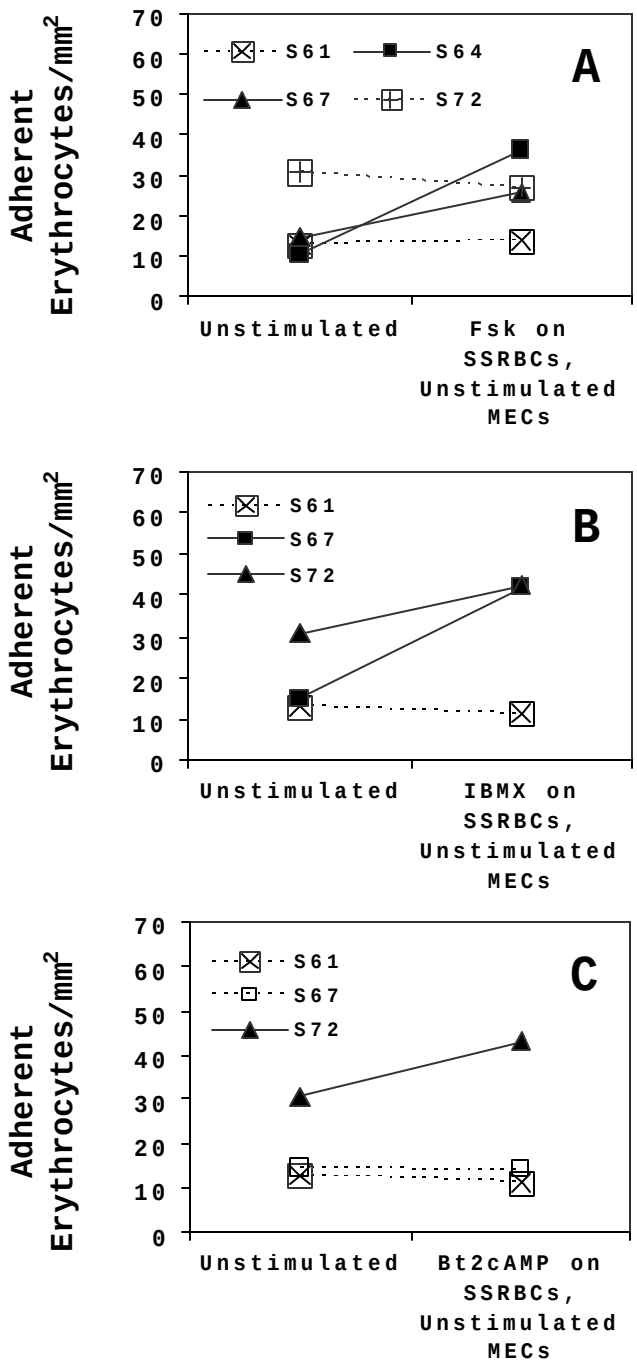


Figure 5.11 Effect of reagents that increase intracellular cAMP in sickle erythrocytes on adhesion to unstimulated MECs. Treatment of SSRBCS with A) 100 μM forskolin (n=4), B) 500 μM IBMX, and C) 500 μM Bt_2cAMP (n=3) prior to perfusion increases adhesion to MECs in some patients after 40 minutes of erythrocyte perfusion. Complete data sets are presented in Tables B.33 and B.34.

5.3.2 Adhesion of SSRBCs Treated with cAMP Elevating Reagents to MECs Stimulated with TNF-α

Figure 5.12 shows the effects of reagents that increase cAMP in sickle erythrocytes on their adhesion to MECs stimulated with TNF-α. Panel A and panel B demonstrate that treatment of sickle erythrocytes with either 100 μM forskolin or 500 μM Bt_2cAMP does not alter adhesion to TNF-α stimulated MECs. Data sets represent the average of several blood samples, because analysis of results from individual blood samples reveals virtually homogeneous results. Only one blood sample responded to treatment with forskolin, resulting in a 1.5-fold increase in adhesion of sickle erythrocytes. None of the samples treated with Bt_2cAMP responded to treatment. Results for individual samples as well as the averages are given in Appendix B, Tables B.35 and B.36.

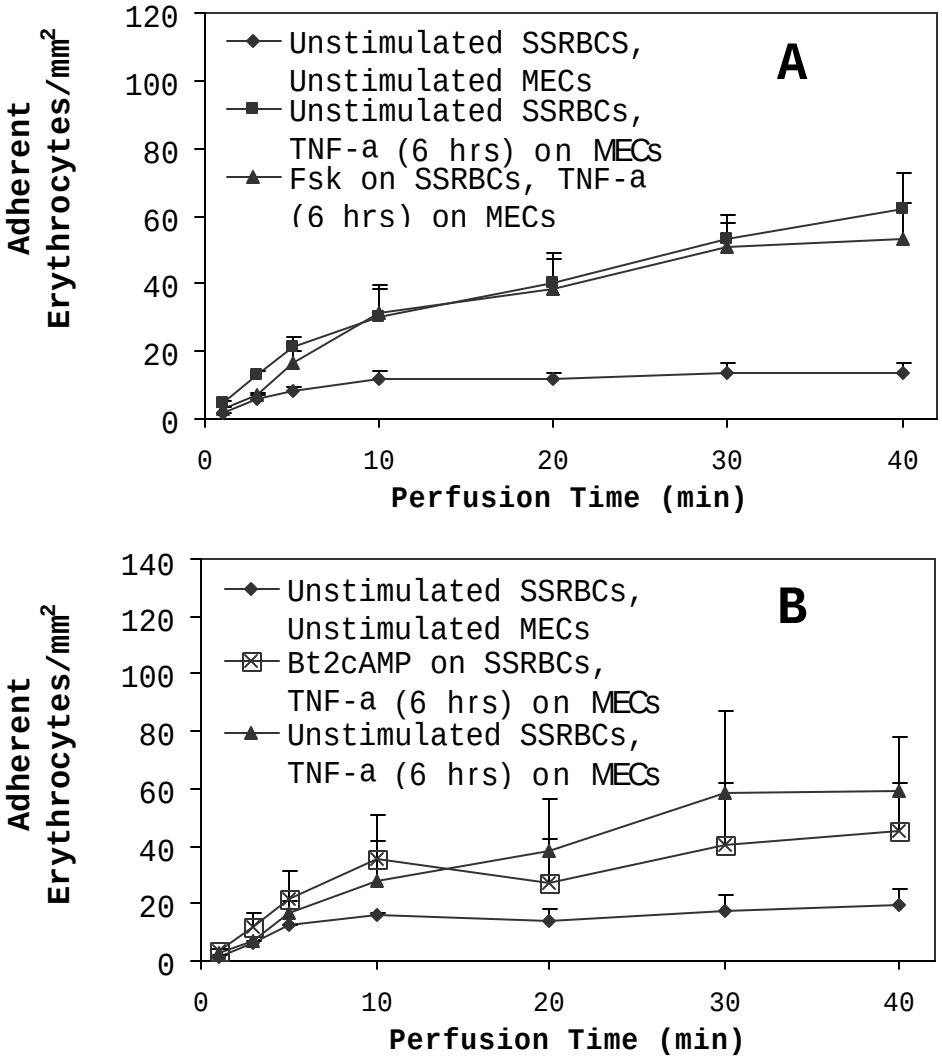


Figure 5.12 Effect of reagents that increase intracellular cAMP in sickle erythrocytes on adhesion to MECs stimulated with TNF-a for 6 hrs. Treatment of SSRBCS with A) 100 μM forskolin (n=4) or B) 500 μM Bt_2cAMP (n=3) prior to perfusion does not affect adhesion. Data for individual blood samples are presented in Tables B.35 and B.36.

5.4 The Effect of Blood Sample Age on Adhesion of Sickle Erythrocytes

Data shown in Chapters 4 and 5 were generated in flow adhesion assays in which blood varied in age from 0 to 56 hrs. Blood age is defined as the time elapsed between drawing of blood from the patient to the time flow adhesion assays are completed. In this section, retrospective analysis of the effect of blood age on sickle erythrocyte adhesion is undertaken.

5.4.1 Effect of Blood Age on Adhesion of Sickle Erythrocytes to Unstimulated and TNF-a Stimulated MECs

Figure 5.13 shows the effect of blood age on sickle erythrocyte adhesion to unstimulated and TNF-α stimulated MECs. It shows all blood samples used to generate data in Chapters 4 through 6 in which adhesion to MECs stimulated with TNF-α for 6 hrs was measured. The values shown are numbers of adherent erythrocytes after 40 minutes of perfusion. The samples are ordered from least to greatest numbers of adhesive erythrocytes to unstimulated MECs, shown by the orange bars. On top of the orange bars, numbers of adhesive erythrocytes to TNF-α stimulated MECs

are shown for each blood sample. The color of the top bar indicates the age of the blood sample when adhesion to unstimulated and TNF-α stimulated MECs was measured. The freshest samples (2-16 hrs old) are shown in green, the middle samples (18-36 hrs) are shown in red, and the oldest samples (44 to 56 hrs) are shown in blue. Qualitatively, Figure 5.13 shows an absence of green, or fresh, samples at the left end of the graph and a higher concentration of fresh samples at the right end of the graph, indicating that sickle erythrocyte adhesion to unstimulated MECs is higher for the freshest samples. This result is confirmed by ANOVA, which indicates a statistically significant difference between numbers of adherent erythrocytes to unstimulated MECs for the green and the blue groups (the freshest versus the oldest samples). The average numbers of adherent sickle erythrocytes for both unstimulated and TNF-α stimulated MECs for each color/age group are given in Figure 5.15, where the statistical difference is indicated.

The reason that sickle erythrocyte adhesion to unstimulated MECs is higher for fresher blood samples is unknown. Fresh erythrocytes from sickle patients have been shown to be more adherent to unstimulated endothelial cells than non-sickle erythrocytes, possibly due to the increased presence of ligands on sickle erythrocytes that bind to the

endothelial cells. However, the receptors and ligands promoting this abnormal adhesion under unstimulated conditions are not well understood. One potential explanation for decreased adhesion of sickle erythrocytes as blood ages is that the ligands on sickle erythrocytes that promote adhesion to unstimulated MECs may be shed into the plasma over time. When the blood samples are washed to isolate the erythrocytes, the shed receptors may be discarded.

As reticulocytes mature, they produce exosomes, the major known function of which is clearing of membrane proteins. One major component these exosomes is the transferrin receptor (Johnstone ***et al.***, 1987). However, a smaller component is $\alpha_4\beta_1$. One study suggests that ***in vitro*** maturation of reticulocytes for 48 hours at 37°C results in almost complete clearing of $\alpha_4\beta_1$ from the surface of reticulocytes. Interestingly, the $\alpha_4\beta_1$ in exosomes remains active, as exosomes containing $\alpha_4\beta_1$ are more adherent to fibronectin (Rieu ***et al.***, 2000). It is not clear that $\alpha_4\beta_1$ is shed from the sickle reticulocytes used in the experiments presented in this document during the storage period, especially given that externalization of membrane proteins appears temperature dependent and the sickle blood

is refrigerated at 4°C prior to use (Orr *et al.*, 1987). It is also not clear that removal of reticulocyte $\alpha_4\beta_1$ would affect adhesion to unstimulated MECs. However, these studies do invite speculation that storage of sickle blood prior to use in flow assays results in exosomal removal of membrane proteins such as $\alpha_4\beta_1$ that are important to sickle cell adhesion.

In Figure 5.14 the same data set as shown in Figure 5.13 is rearranged to show increasing sickle erythrocyte adhesion to MECs stimulated with TNF-α for 6 hrs. Qualitatively, no particular color trend is evident, and ANOVA results indicate no statistical difference between numbers of adherent erythrocytes to TNF-α stimulated MECs between the three color/age groups.

5.4.2 Effect of Blood Age on cAMP Inhibition of Sickle Erythrocyte Adhesion to TNF-a Stimulated MECs

Only minimal data exist to test whether or not blood age affects the ability of cAMP elevating agents to inhibit sickle erythrocyte adhesion. Table 5.2 shows that 10 blood samples were used to test the effects of forskolin treatment of MECs on TNF-α induced adhesion. Of the ten samples, two were used within 16 hrs, and the other eight

were used within 56 hours of drawing. The average for the two fresh samples shows higher unstimulated sickle erythrocyte adhesion and higher percent inhibition of adhesion due to forskolin treatment than the average of the eight older samples. On the other hand, eight blood samples were used to test the effects of IBMX and Bt_2cAMP on sickle cell adhesion, one of which was used within 16 hrs of drawing and the others of which were used within 36 hrs. These data demonstrate that adhesion and percent inhibition were similar regardless of blood age. Given the small sample size of the freshest blood samples, whether or not blood age affects the ability of cAMP-elevating reagents to inhibit sickle erythrocyte adhesion cannot be determined conclusively.

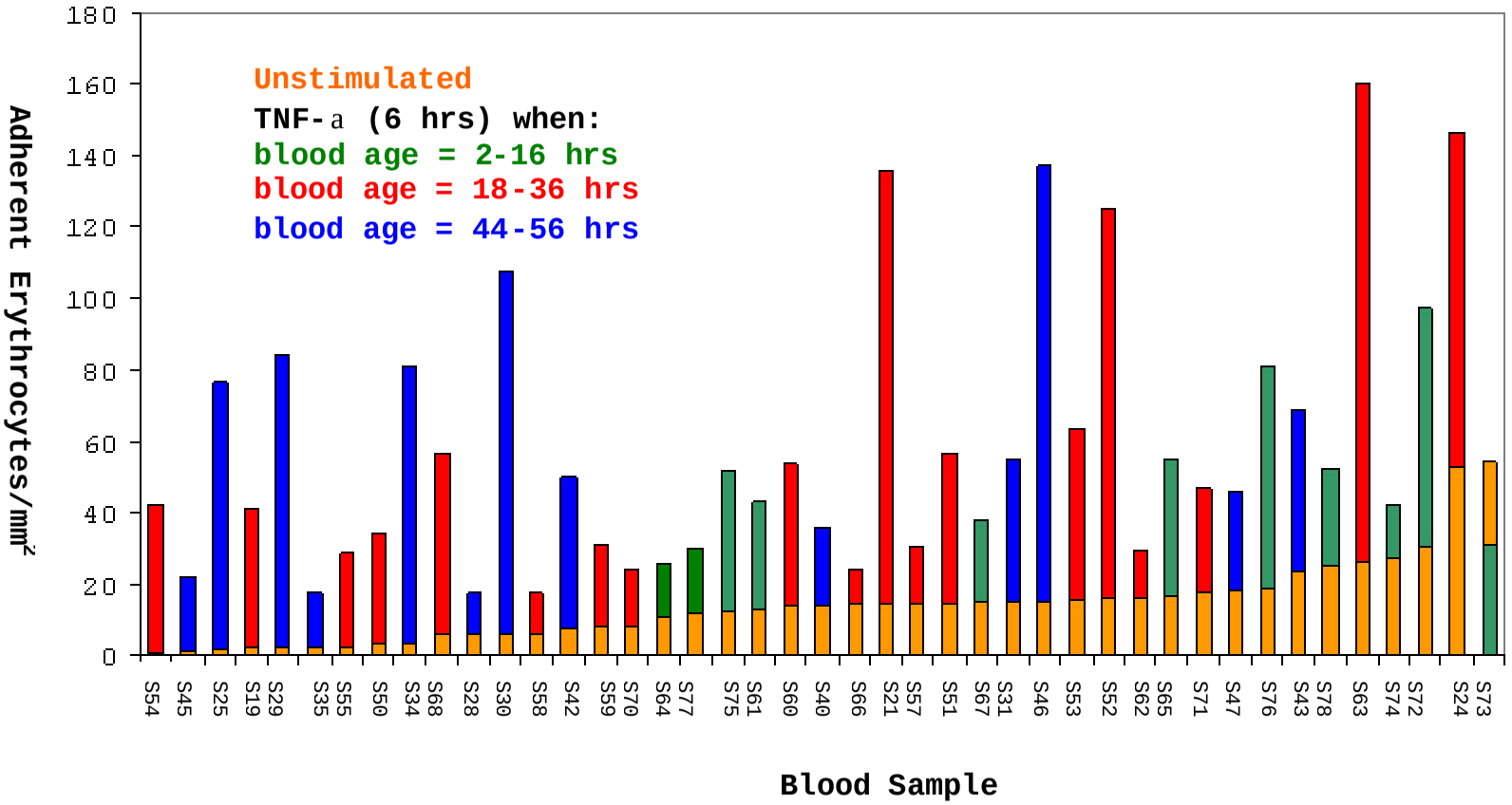


Figure 5.13 Effect of blood age on sickle erythrocyte adhesion to unstimulated and TNF-a stimulated MECs. Adhesion of sickle erythrocytes to unstimulated and TNF-α stimulated MECs arranged by increasing adhesion to unstimulated MECs. Color of top bars indicates the age of the blood sample when flow adhesion assay was completed. Data for individual blood samples are also presented in Table B.37.

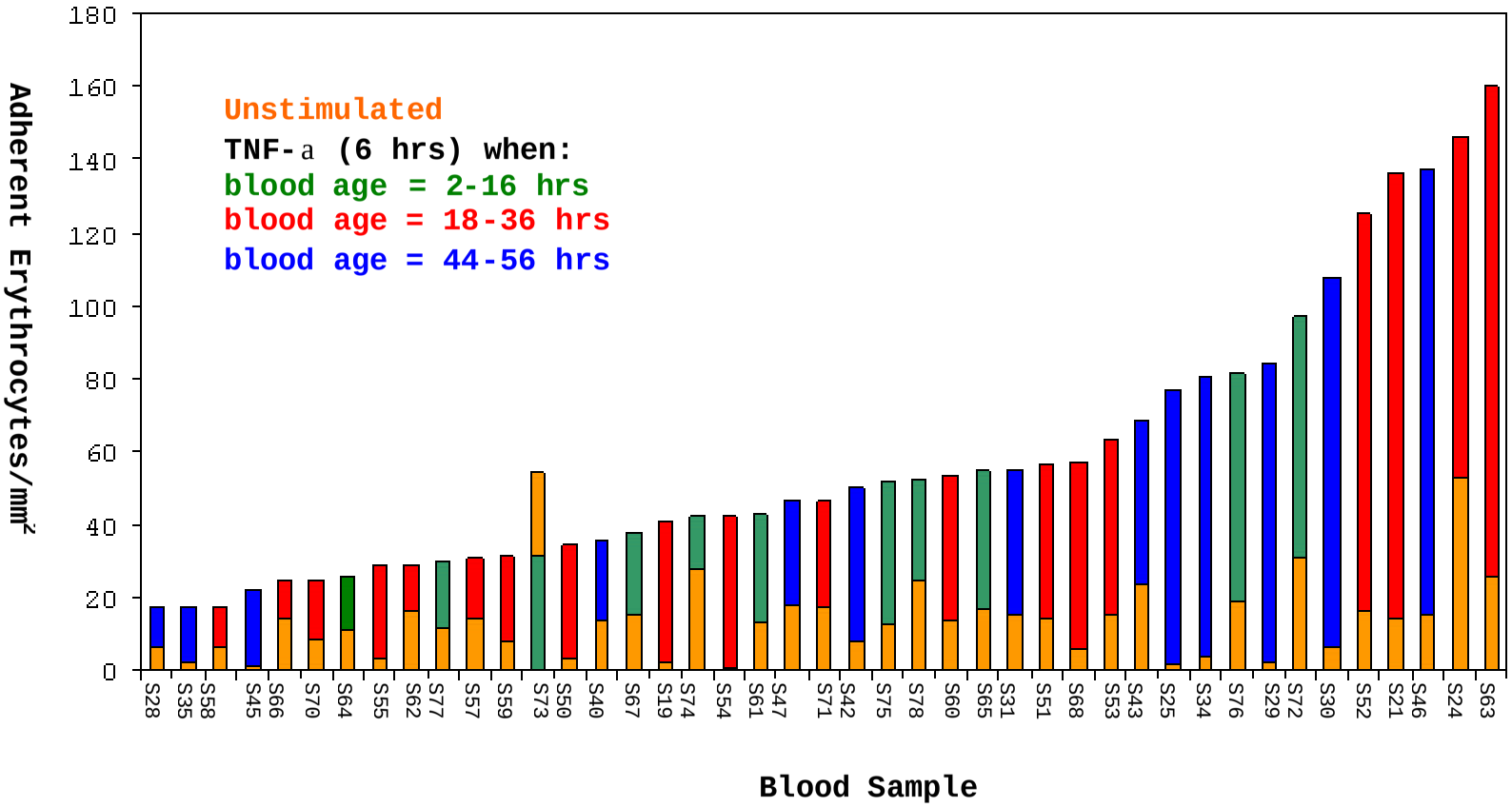


Figure 5.14 Effect of blood age on sickle erythrocyte adhesion to unstimulated and TNF-a stimulated MECs (rearranged). Adhesion of sickle erythrocytes to unstimulated and TNF-α stimulated MECs arranged by increasing adhesion to TNF-α stimulated MECs. Color of top bars indicates the age of the blood sample when flow adhesion assay was completed. Data for individual blood samples are also presented in Table B.37.

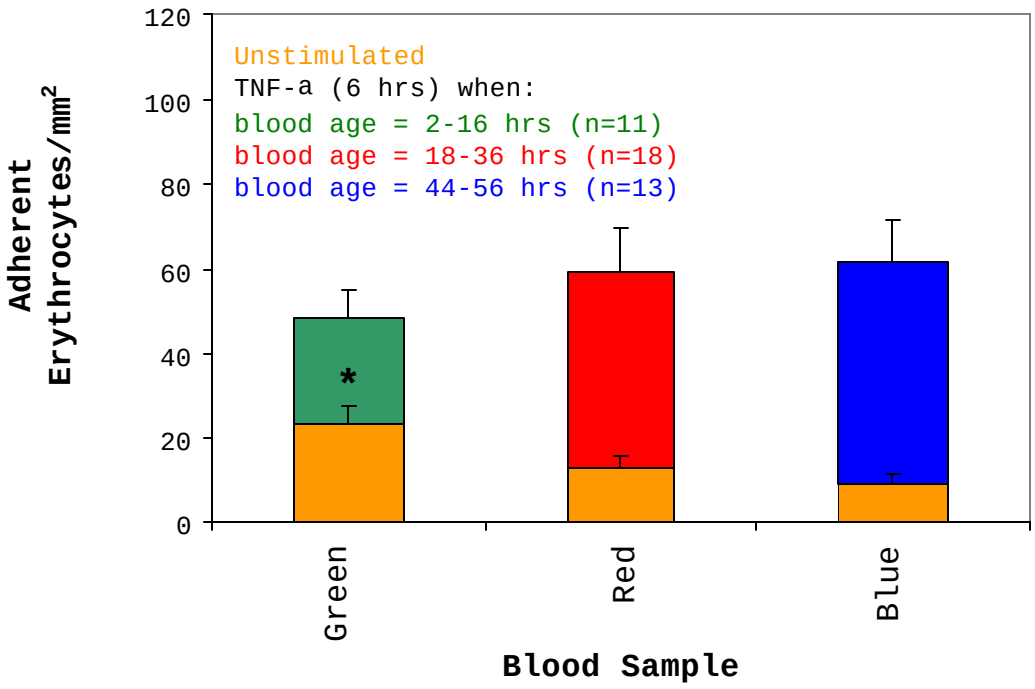


Figure 5.15 Average adhesion to unstimulated and TNF-a stimulated MECs as a function of blood age. The color of top bars indicates the age of the blood sample when flow adhesion assay was completed. *Indicates statistical difference from unstimulated adhesion when blood age was 44 to 56 hrs. Data for individual blood samples are also presented in Table B.37.

Table 5.2 Effect of blood age on percent inhibition in sickle erythrocyte adhesion caused by cAMP-elevating agents. Data for individual blood samples are given in Tables B.28 and B.29.

	Blood Age		
Endothelial Treatment	**18-56 hrs (n=8)**	**2-16 hrs (n=2)**	**All Samples (n=10)**
Unstimulated	13.8	21.8	15.4
Fsk (pre + cont) + TNF-^{a} (6 hrs)	47.0	30.2	43.7
TNF-^{a} (6 hrs)	87.1	70.0	83.7
% Inhibition			
Fsk (pre + cont) + TNF-^{a} (6 hrs)	54.7	82.5	58.6

	Blood Age		
Endothelial Treatment	**18-36 hrs (n=7)**	**2-16 hrs (n=1)**	**All Samples (n=8)**
Unstimulated	8.9	13.0	9.4
IBMX (pre + cont) + TNF-^{a} (6 hrs)	22.6	23.7	22.8
Bt2cAMP (pre + cont) + TNF-^{a} (6 hrs)	22.6	24.7	22.9
TNF-^{a} (6 hrs)	47.1	42.7	46.5
% Inhibition			
IBMX (pre + cont) + TNF-^{a} (6 hrs)	64.0	64.0	64.0
Bt2cAMP (pre + cont) + TNF-^{a} (6 hrs)	64.0	60.6	63.7

5.4.3 Effect of Blood Age on Adhesion of Forskolin Treated Sickle Erythrocytes to MECs

As shown in Table 5.3, treatment of sickle erythrocytes with forskolin prior to perfusion does not affect adhesion to unstimulated MECs when the sickle erythrocytes are 44 to 56 hours old (n=4). However, as noted in Section 5.3.1, treatment of sickle erythrocytes that are less than 16 hrs old with forskolin induces

adhesion to unstimulated MECs two of four samples tested. These results are reflected in the increased average adhesion for forskolin-treated erythrocytes that are 2-16 hrs old as compared to untreated sickle erythrocytes of the same age. Also, it is worth noting that the adhesion of unstimulated sickle erythrocytes is higher for fresh samples as compared to older samples, as noted in Section 5.4.1. Adhesion of fresh sickle erythrocytes is also increased by treatment with IBMX or Bt_2cAMP.

Table 5.3 Effect of blood age on sickle erythrocyte adhesion induced by treatment of erythrocytes with reagents that increase intracellular cAMP.

	Blood Age		
Erythrocyte Treatment	**44-56 hrs (n=4)**	**2-16 hrs (n=4)**	**All Samples (n=8)**
Untreated	9.8	17.3	13.5
Fsk	9.4	25.7	17.6

	Blood Age		
Erythrocyte Treatment	**44-56 hrs (n=0)**	**2-16 hrs (n=3)**	**All Samples (n=3)**
Untreated		19.5	19.5
IBMX		32.0	32.0
Bt2cAMP		22.9	22.9

5.5 Chapter Summary

The data presented in Chapter 5 demonstrate that inhibition of endothelial adhesion molecule expression and sickle erythrocyte adhesion is achieved by elevation of endothelial intracellular cAMP levels. The expression of VCAM-1 and E-selectin, but not ICAM-1 on TNF-α stimulated MECs is reduced by treatment of MECs with Forskolin, IBMX, or Bt_2cAMP, which increase endothelial cAMP levels by different mechanisms. The degree of reduction depends primarily on the length of treatment time with cAMP-elevating reagents and, to a lesser extent, on the concentration of reagent used. While significant reductions in adhesion molecule expression are achieved by pretreatment of MECs with cAMP-elevating reagents, the largest reductions in expression are seen when the reagents are present both prior to and during TNF-α stimulation.

Pretreatment and continuous presence of cAMP-elevating reagents during TNF-α stimulation of MECs also results in significantly decreased sickle erythrocyte adhesion. Adhesion is not, however, completely attenuated, with percent inhibition of adhesion varying from 59% to 64%, depending on the cAMP-elevating reagent used. Incomplete

inhibition of sickle erythrocyte adhesion may result from incomplete inhibition of adhesion molecule expression.

Experiments in which sickle erythrocytes, rather than endothelial cells, are treated with cAMP-elevating reagents prior to perfusion show that adhesion to TNF-α stimulated MECs is not altered. However, adhesion to unstimulated MECs is increased for some blood samples. These results are consistent with other reports in literature (Zennadi et al., 2004).

In addition to the effects of cAMP-elevating reagents on sickle erythrocyte adhesion, the preceding chapter also addresses the effect of blood age after drawing from the patient. Retrospective analysis demonstrates that adhesion of sickle erythrocyte to unstimulated MECs is higher for fresher blood samples than for older ones. Levels of TNF-α induced adhesion are not significantly altered. It is unclear whether or not blood age affects the ability of cAMP elevating reagents on MECs to inhibit TNF-α induced sickle erythrocyte adhesion. However, it appears as though treatment of some fresh sickle erythrocytes with cAMP-elevating reagents increases adhesion to unstimulated MECs, whereas older blood samples are not affected by treatment.

Together these data demonstrate that cAMP is an effective inhibitor of TNF-α induced adhesion molecule expression and sickle erythrocyte adhesion. While the data presented in this chapter were obtained under *in vitro* conditions, it is also important to consider what is known from literature about *in vivo* effects of elevated cAMP in animals and humans with and without sickle cell anemia. Pentoxifylline, a methyl xanthine derivative, has been used to treat peripheral vascular disease in humans for over 30 years, and the generally accepted mechanism of its action is increasing levels of intracellular cAMP by inhibition of phosphodiesterases (Windmeier & Gressner, 1997). Clinical trials in which the drug was administered to sickle patients either three times daily or at presentation of pain episodes showed the groups taking pentoxifylline to have fewer patients experiencing pain episodes, fewer total pain episodes, shorter duration of pain events, and shorter duration of inpatient treatment than groups on placebo (Manrique, 1987; Teuscher *et al.*, 1989). The therapeutic benefit of this drug is thought to be primarily due to reduced platelet aggregation and blood viscosity (Windmeier & Gressner, 1997), as well as increased erythrocyte flexibility caused in part by inhibition of cell dehydration and sickling (Ambrus *et al.*, 1988; Stuart *et*

al., 1987). However, administration of pentoxifylline to non-sickle patients has also resulted in decreases in circulating TNF-α and soluble VCAM-1, E-selectin, and ICAM-1 in patient plasma (Boldt *et al.*, 1996; Noel *et al.*, 2000), implying that pentoxifylline suppresses endothelial cell activation. While similar studies have not been conducted in sickle patients, the data presented in this chapter suggest that one of the beneficial effects of pentoxifylline in sickle cell anemia may be reduction in adhesion molecule expression and sickle erythrocyte adhesion.

In addition to regulating expression of endothelial adhesion molecules, elevated cAMP also causes vasodilation in rabbit spinal arterioles (Yashiro & Ohhashi, 2002) and transgenic sickle mice (Kaul et al., 2000), a response generally attributed to production of endothelial nitric oxide. The following chapter explores the role of nitric oxide in regulating endothelial adhesion molecule expression and sickle erythrocyte adhesion.

CHAPTER 6

REGULATION OF CYTOKINE INDUCED ADHESION MOLECULE EXPRESSION AND SICKLE ERYTHROCYTE ADHESION BY ELEVATION OF ENDOTHELIAL NITRIC OXIDE

As outlined in Section 2.7, nitric oxide is a soluble gas synthesized upon activation of nitric oxide synthase in endothelial cells. The most notable physiologic effect of nitric oxide release is vasodilation. However, nitric oxide also appears to have anti-coagulatory and anti-inflammatory properties (Gladwin & Schechter, 2001). Nitric oxide bioavailability in sickle patients, especially men, is reduced (Gladwin et al., 2003), suggesting that strategies to improve NO bioavailability may be of benefit to sickle patients.

Several studies indicate that nitric oxide inhibits inflammation-induced VCAM-1 (and possibly E-selectin) expression on endothelial cells. The ability of NO to regulate adhesion molecule expression and sickle erythrocyte adhesion is the subject of this chapter.

6.1 Regulation of Endothelial Adhesion Molecule Expression by Reagents that Increase Nitric Oxide

Panel A of Figure 6.1 shows adhesion molecule expression on unstimulated MECs (white bars), MECs stimulated with TNF-α for 6 hrs (black bars), and MECs treated with 500 μM sodium nitroprusside (a nitric oxide donor) for 30 minutes prior to and during 6 hrs of TNF-α stimulation (gray bars). Sodium nitroprusside significantly inhibits TNF-a induced VCAM-1 expression, but not E-selectin or ICAM-1 expression. These results are mimicked by treatment of MECs with 500 μM DETA-NO, a different nitric oxide donor (Panel B). Several other reports in literature confirm that nitric oxide partially, but not completely, inhibits VCAM-1 mRNA and protein expression (De Caterina et al., 1995; Khan et al., 1996; Spiecker et al., 1997). However, literature regarding the effects of NO on E-selectin and ICAM-1 is conflicting, with some authors suggesting an inhibition in expression (De Caterina et al., 1995) and others suggesting no effect (Khan et al., 1996). Figure 6.1 indicates a small, statistically insignificant inhibition. To ensure that the ability of these reagents to inhibit VCAM-1 expression is not due to cell death, flurorescent cell viability assays

were performed as described above. Light and confocal microscopy photographs of MECs treated with NO donors and TNF-α are given in Appendix C.

That VCAM-1, but not E-selectin, expression is significantly inhibited by nitric oxide indicates a divergence in the cell signaling cascades leading to expression of these two receptors. As more is learned about the transcription factors and co-factors that regulate receptor transcription, it may be possible to locate the point of divergence and tailor receptor expression or inhibition therapies to specific molecules.

In addition to their effects on TNF-α induced adhesion molecule expression, the effects of treatment of endothelial cells with SNP and DETA-NO alone (without TNF-α) are of interest. Table 6.1 shows that for the adhesion molecules tested, expression is not upregulated by treatment of MECs with reagents that increase NO.

The data presented in this section suggest that increasing endothelial nitric oxide with SNP or DETA-NO will inhibit TNF-α induced sickle erythrocyte adhesion but will not affect unstimulated adhesion. The results presented in Section 6.2 test this hypothesis.

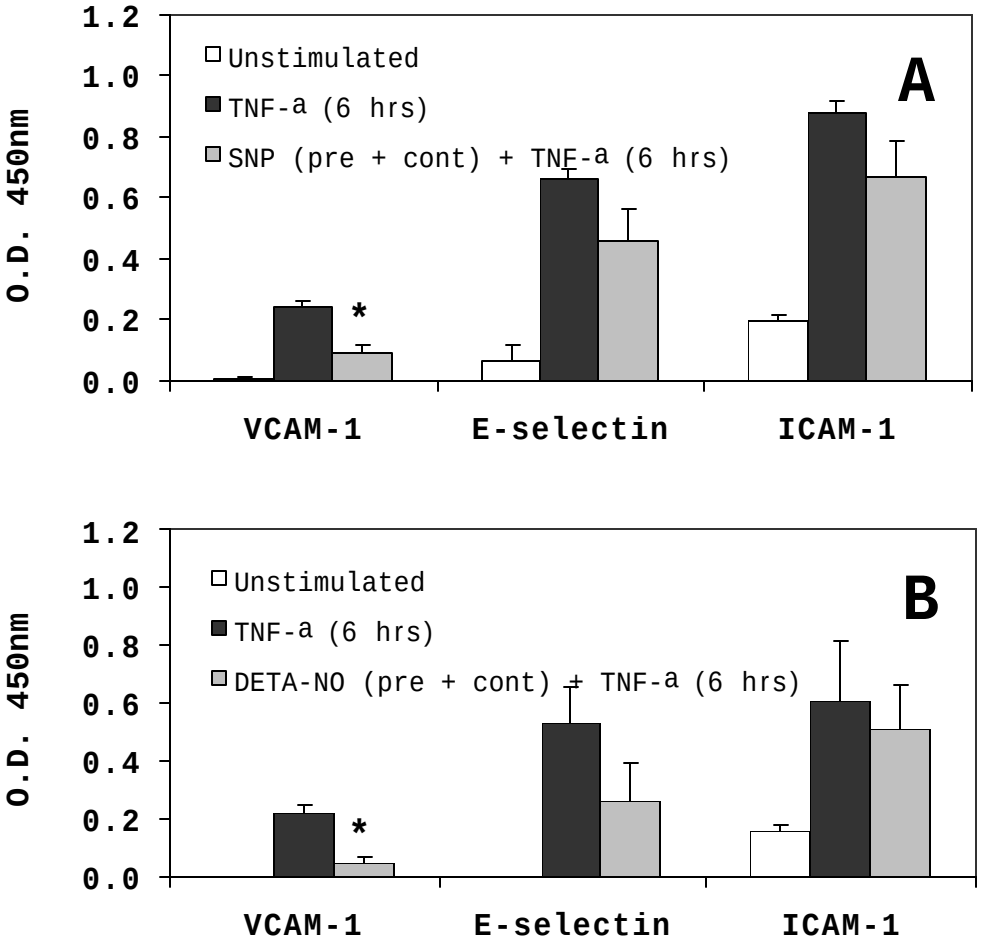


Figure 6.1 Inhibition of TNF-a induced adhesion molecule expression by reagents that increase intracellular nitric oxide. VCAM-1 expression, but not E-selectin or ICAM-1 expression, is significantly inhibited by pretreatment and continuous presence of A) SNP (n=6) or B) DETA-NO (n=4) during 6 hrs of TNF-α stimulation. *Indicates statistically significant difference from MECs treated with only TNF-α. Data for individual experiments are presented in Tables B.38 and B.39.

Table 6.1 Adhesion molecule expression on MECs treated with SNP or DETA-NO alone. Adhesion molecule expression is not significantly upregulated by treatment of MECs with SNP or DETA-NO alone for 6.5 hrs (n=3). Data for individual experiments are presented in Tables B.40 and B.41.

Endothelial Treatment	VCAM-1	E-selectin	ICAM-1
Unstimulated	0.01 ± 0.01	0.02 ± 0.02	0.18 ± 0.01
SNP	0.01 ± 0.01	0.05 ± 0.05	0.13 ± 0.03
TNF-α	0.15 ± 0.06	0.48 ± 0.14	0.68 ± 0.13

Endothelial Treatment	VCAM-1	E-selectin	ICAM-1
Unstimulated	0.01 ± 0.01	0.03 ± 0.03	0.18 ± 0.06
DETA-NO	0.02 ± 0.02	0.04 ± 0.04	0.33 ± 0.07
TNF-α	0.19 ± 0.06	0.58 ± 0.07	0.70 ± 0.07

6.2 Effect of Reagents that Increase Endothelial Nitric Oxide on Sickle Erythrocyte Adhesion

The data presented in Section 6.1 demonstrate that NO donors partially inhibit endothelial expression of VCAM-1 induced by TNF-α. To test their effects on sickle erythrocyte adhesion, MECs were treated with 500 μM SNP or 500 μM DETA-NO for 30 minutes prior to and during 6 hrs of TNF-α stimulation, mimicking the conditions outlined in Section 6.1. Panel A of Figure 6.2 demonstrates that SNP inhibits TNF-α induced adhesion by 71% after 40 minutes of erythrocyte perfusion. Similar results are obtained with DETA-NO, which inhibits adhesion by 79%.

Like the results obtained when MECs were treated with cAMP, inhibition of TNF-α induced adhesion by NO donors is partial, but not complete. Incomplete inhibition of sickle erythrocyte adhesion may be due to incomplete inhibition of adhesion molecule expression on MECs, since VCAM-1 is only partially reduced and E-selectin expression is not changed by NO donors. Partial inhibition of adherence of leukocytes (De Caterina et al., 1995; M. Z. Jiang *et al.*, 2005), non-sickle erythrocytes (Space et al., 2000), and *Plasmodium falciparum*-infected erythrocytes (Serirom et al., 2003) to cytokine stimulated endothelial cells by exogenous NO donors has been observed by others *in vitro*.

In addition to partial inhibition of endothelial receptors by NO donors, the age of the blood samples, or time elapsed between drawing from the patient and perfusion over MECs may affect adhesion. This possibility is further discussed in Section 6.4.

While the data in Figure 6.2 demonstrate that treatment of endothelial cells with NO donors inhibits TNF-α induced sickle erythrocyte adhesion, the data in Figure 6.3 show that treatment of MECs with NO donors alone (without TNF-α) for 6.5 hrs does not affect adhesion to otherwise untreated endothelial cells. That is,

unstimulated adhesion of erythrocytes, which is elevated in sickle patients when compared to non-sickle patients, is not affected by NO donors under conditions of continuous flow. These results are in conflict with those of Space, et al., who show the nitric oxide donor DETA-NO decreases adhesion of sickle erythrocytes to unstimulated endothelial cells by 54% (Space et al., 2000). However, three main differences in experimental protocols may explain the results. First, Space, et al. use a higher concentration of DETA-NO than is used to produce the results of Figure 6.3 (2000 μM versus 500 μM). This higher concentration may result in an effect not seen with a lower concentration. Second, numbers of adherent erythrocytes in this work are counted under conditions of continous flow, whereas Space, et al. work under conditions of intermittent flow. As discussed in Section 2.4.3, adhesion of sickle erythrocytes is higher under static conditions than under flow. This may account for the high numbers of adherent erythrocytes to unstimulated MECs that Space, et al. show (387 ± 111 erythrocytes/mm^2 versus 17 ± 3 erythrocytes/mm^2) and for the ability of the NO donor DETA-NO to inhibit this adhesion. Finally, it is important to note that the data presented in Figure 6.3 were generated under conditions in which only the MECs, and not the erythrocytes, are treated with an NO

donor, whereas Space, et al. treat both the MECs and the erythrocytes with DETA-NO. The effect of treatment of sickle erythrocytes with NO donors on their adhesion to endothelial cells is the subject of Section 6.3.

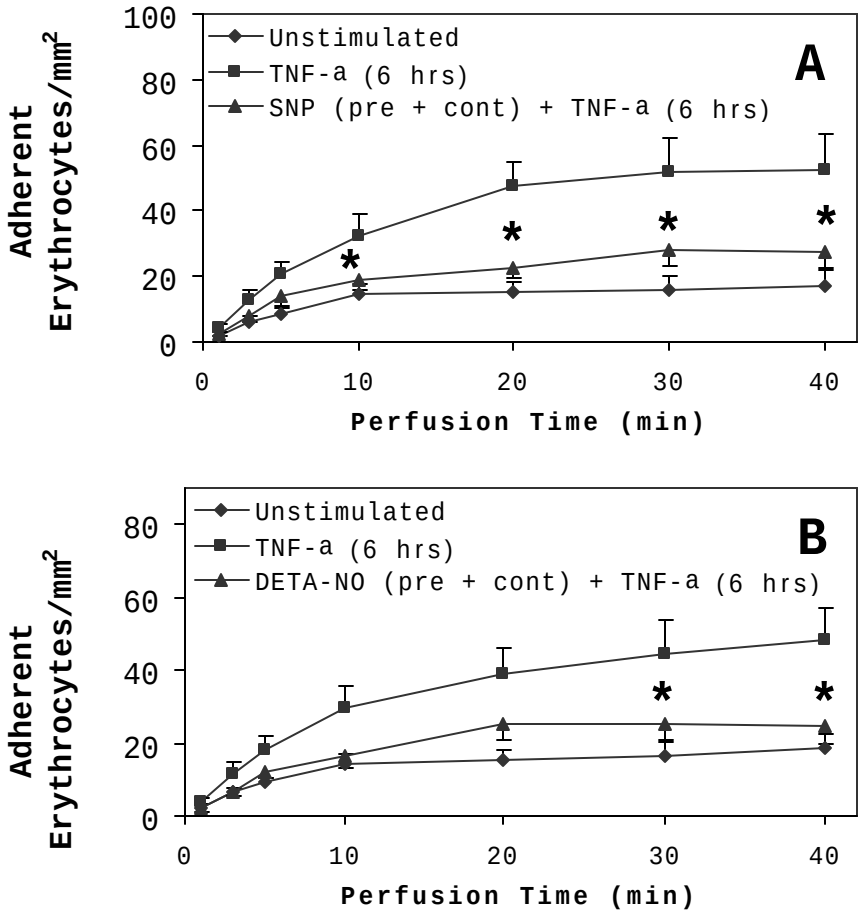


Figure 6.2 Inhibition of TNF-a induced sickle erythrocyte adhesion by reagents that increase intracellular nitric oxide. Treatment of MECs with A) 500 μM SNP (n=10) or B) 500 μM DETA-NO (n=11) before and during 6 hrs of TNF-α stimulation inhibits adhesion of sickle erythrocytes. *Indicates statistically significant difference from MECs treated with only TNF-α. Data for individual blood samples are presented in Tables B.42 and B.43.

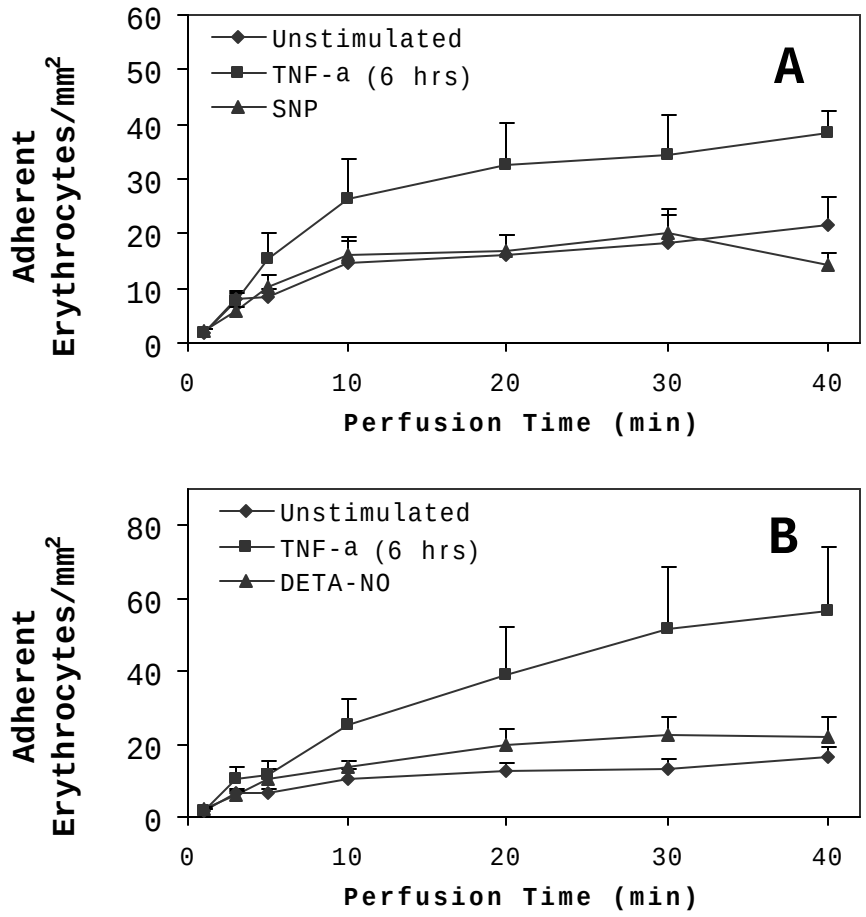


Figure 6.3 Effect of reagents that increase endothelial intracellular NO on sickle erythrocyte adhesion. A) 500 μM SNP (n=8) and DETA-NO (n=7) alone (without TNF-α) do not affect sickle erythrocyte adhesion to MECs. Data for individual blood samples are presented in Tables B.44 and B.45.

6.3 Treatment of Sickle Erythrocytes with Nitric Oxide Donors

Figure 6.4 shows adhesion of sickle erythrocytes treated with either 500 μM SNP (Panel A) or DETA-NO (Panel B) for 40 minutes prior to perfusion over unstimulated MECs. The data demonstrate that treatment of sickle erythrocytes with NO donors does not affect their adhesion to unstimulated endothelial cells. Figure 6.5 shows adhesion of sickle erythrocytes treated with NO donors to MECs stimulated with TNF-α for 6 hrs. Treatment of erythrocytes with SNP inhibits adhesion 28% (Panel A), while treatment with DETA-NO inhibits adhesion by 50% (Panel B), although these results are not statistically significant.

Little information exists in the literature to support or explain the results shown in Figure 6.5. Space et. al. report inhibition of TNF-α induced adhesion when both endothelial cells and erythrocytes are treated with the NO donor DETA-NO, but it is not clear whether the effect is on the endothelium, the erythrocytes, or both (Space et al., 2000). However, treatment of neutrophils with reagents that inhibit nitric oxide induces expression of $\alpha_4\beta_1$, which

binds to VCAM-1, possibly through mobilization of neutrophil granules and cytoskeletal rearrangement (Conran et al., 2003). It is not clear, though, that NO donors affect $\alpha_4\beta_1$ expression on neutrophils or that the response to NO extends to erythrocytes. In addition, patients taking hydroxyurea, which generates NO, show fewer erythrocytes expressing $\alpha_4\beta_1$ and fewer $\alpha_4\beta_1$ molecules per erythrocyte than sickle patients not taking hydroxyurea (Styles et al., 1997). Although this effect may not result from NO exposure, it invites speculation that one beneficial effect of NO for sickle patients may be reduction in $\alpha_4\beta_1$ expression on sickle erythrocytes and reduced sickle erythrocyte adhesion.

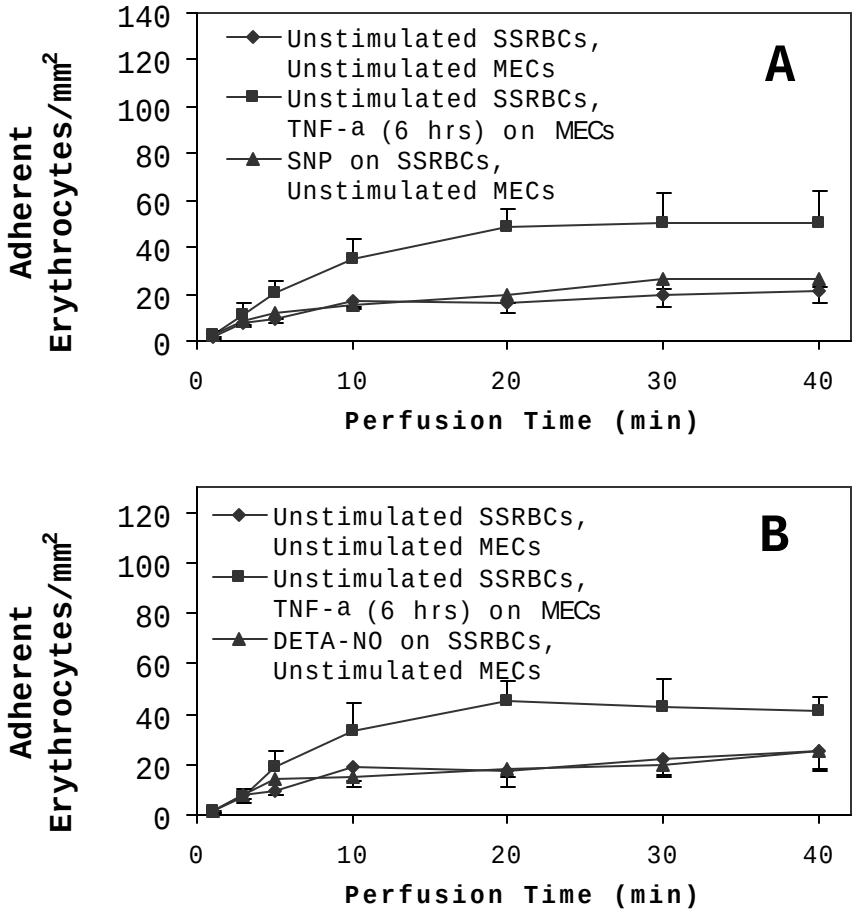


Figure 6.4 Adhesion of sickle erythrocytes treated with NO donors to unstimulated MECs. Treatment of sickle erythrocytes with A) 500 μM SNP (n=7) or B) 500 μM DETA-NO (n=5) prior to perfusion does not affect adhesion to unstimulated MECs. Data for individual blood samples are presented in Tables B.46 and B.47.

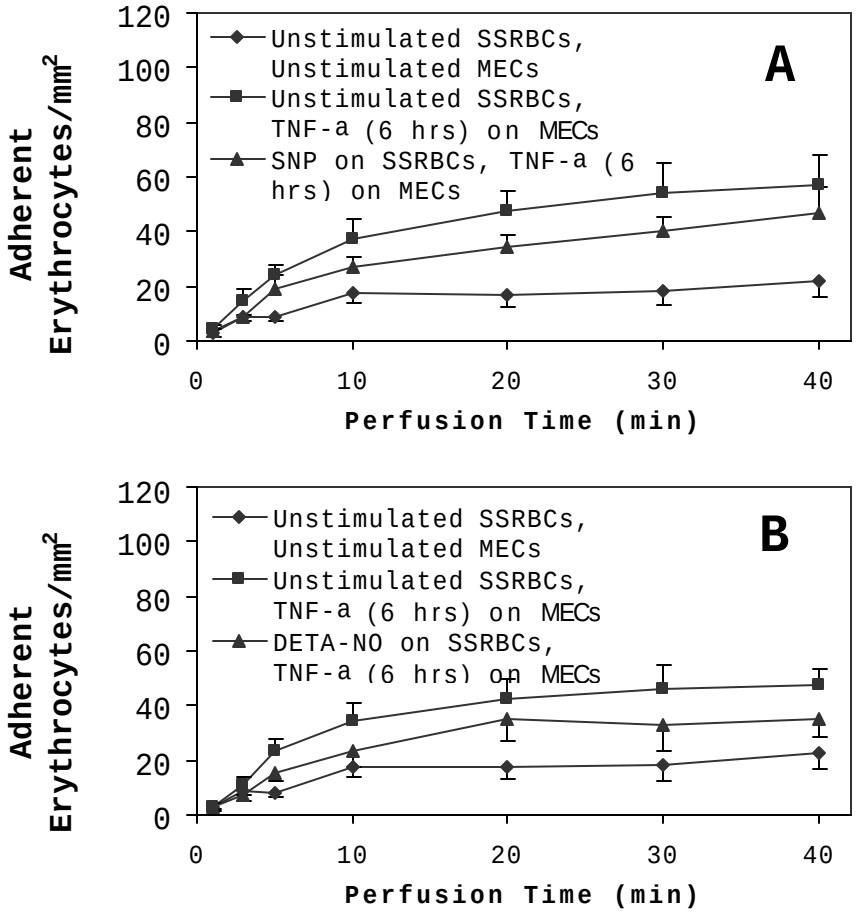


Figure 6.5 Adhesion of sickle erythrocytes treated with NO donors to TNF-a stimulated MECs. Treatment of sickle erythrocytes with A) 500 μM SNP (n=8) or B) 500 μM DETA-NO (n=7) prior to perfusion over TNF-α stimulated MECs. Data for individual blood samples are presented in Tables B.48 and B.49.

6.4 Effect of Blood Age on Sickle Erythrocyte Adhesion

Data presented in Chapter 6 were obtained in flow assays in which blood samples were 2 to 36 hours old at the time of erythrocyte perfusion. Here the effect of blood age on sickle erythrocyte adhesion is discussed.

6.4.1 Sickle Erythrocyte Adhesion to MECs Treated with Reagents that Increase Nitric Oxide

Table 6.2 shows numbers of adherent erythrocytes to unstimulated and TNF-α stimulated MECs and also to MECs exposed to nitric oxide donors before and during TNF-α stimulation after 40 minutes of erythrocyte perfusion. Blood samples are categorized by age, and the average of all blood samples of a given age is shown. The far right column represents the average of all blood samples tested. The data show that numbers of adherent erythrocytes do not vary with blood age for MECs stimulated with TNF-α, SNP + TNF-α, or DETA-NO + TNF-α. However, unstimulated adhesion tends to be higher for younger blood samples than older ones, consistent with the data in Section 5.4.

Table 6.3 shows adhesion of sickle erythrocytes to MECs treated with SNP or DETA-NO alone. The fold change in

adhesion due to treatment with these reagents as compared to unstimulated MECs varies from 0.6 to 1.5 depending on the reagent and age of blood. However, these results do not change the overall conclusion that nitric oxide donors alone on MECs do not significantly affect sickle erythrocyte adhesion.

Table 6.2 Blood age and adhesion of sickle erythrocytes to MECs stimulated with TNF-a and nitric oxide donors. Numbers of adherent erythrocytes after 40 minutes of perfusion are shown. Average data for all blood samples are represented graphically in Figure 6.2, and data for individual blood samples are presented in Tables B.42 and B.43.

	Blood Age		
Endothelial Treatment	**18-36 hrs (n=6)**	**2-16 hrs (n=4)**	**All (n=10)**
Unstimulated	9.8	28.2	17.1
TNF-α (6 hrs)	53.4	51.6	52.7
SNP (pre + cont) + TNF-α (6 hrs)	26.7	28.6	27.4
% Inhibition	**61.2**	**98.6**	**71.0**

	Blood Age		
Endothelial Treatment	**18-36 hrs (n=7)**	**2-16 hrs (n=4)**	**All (n=11)**
Unstimulated	13.5	28.2	18.9
TNF-α 6 hrs	46.1	51.6	48.1
DETA-NO (pre + cont) + TNF-α (6 hrs)	24.4	25.8	24.9
% Inhibition	**66.6**	**110.3**	**79.3**

Table 6.3 Blood age and adhesion of sickle erythrocytes to MECs treated with nitric oxide donors. Numbers of adherent erythrocytes after 40 minutes of perfusion are shown. Average data for all blood samples are represented graphically in Figure 6.3, and data for individual blood samples are presented in Tables B.44 and B.45.

	Blood Age		
Endothelial Treatment	**18-36 hrs (n=3)**	**2-16 hrs (n=5)**	**All (n=8)**
Unstimulated	13.9	26.1	21.5
TNF-α (6 hrs)	33.4	41.6	38.5
SNP	11.5	16.0	14.3
Fold change	**0.8**	**0.6**	**0.7**

	Blood Age		
Endothelial Treatment	**18-36 hrs (n=4)**	**2-16 hrs (n=3)**	**All (n=7)**
Unstimulated	16.9	16.2	16.6
TNF-α (6 hrs)	65.1	44.8	56.4
DETA-NO	19.8	25.0	22.0
Fold change	**1.2**	**1.5**	**1.3**

6.4.2 Adhesion of Sickle Erythrocytes Treated with Nitric Oxide Donors

Table 6.4 shows the same data presented in Figure 6.4 for 40 minutes of perfusion, but samples are separated by blood age. While it appears as though sickle erythrocyte adhesion to unstimulated MECs is higher for younger blood samples than for older ones (regardless of whether or not the sickle erythrocytes are treated with NO donors), the overall conclusion that increased NO in sickle erythrocytes

does not affect adhesion to unstimulated MECs remains the same.

Table 6.5 shows the data presented in Figure 6.5, but again samples are divided by blood age. Significant differences between younger and older blood samples cannot be determined due to the small number of older blood samples, and it is unclear whether blood age affects the ability of nitric oxide to inhibit TNF-α induced sickle erythrocyte adhesion.

Table 6.4 Blood age and adhesion of sickle erythrocytes treated with nitric oxide donors to MECs. Numbers of adherent erythrocytes after 40 minutes of perfusion are shown. Average data for all blood samples are represented graphically in Figure 6.4, and data for individual blood samples are presented in Tables B.46 and B.47.

	Blood Age		
Endothelial Treatment	**18-36 hrs (n=3)**	**2-16 hrs (n=4)**	**All (n=7)**
Unstimulated SSRBCs, Unstimulated MECs	13.6	27.7	21.7
Unstimulated SSRBCs, TNF-α (6 hrs) on MECs	58.4	45.1	50.8
SNP on SSRBCs, Unstimulated MECs	21.6	30.2	26.5
Fold change	**1.6**	**1.1**	**1.2**

	Blood Age		
Endothelial Treatment	**18-36 hrs (n=2)**	**2-16 hrs (n=3)**	**All (n=5)**
Unstimulated SSRBCs, Unstimulated MECs	15.4	31.4	25.0
Unstimulated SSRBCs, TNF-α (6 hrs) on MECs	39.6	41.8	41.0
DETA-NO on SSRBCs, Unstimulated MECs	15.3	32.0	25.3
Fold change	**1.0**	**1.0**	**1.0**

Table 6.5 Blood age and adhesion of sickle erythrocytes treated with NO donors to TNF-a stimulated MECs. Numbers of adherent erythrocytes after 40 minutes of perfusion are shown. Average data for all blood samples are represented graphically in Figure 6.5, and data for individual blood samples are presented in Tables B.48 and B.49.

	Blood Age		
Endothelial Treatment	**18-36 hrs (n=2)**	**2-16 hrs (n=6)**	**All (n=8)**
Unstimulated SSRBCs, Unstimulated MECs	12.0	24.9	21.7
Unstimulated SSRBCs, TNF-α (6 hrs) on MECs	83.5	48.1	57.0
SNP on SSRBCs, TNF-α (6 hrs) on MECs	67.6	40.0	46.9
% Inhibition	**22.3**	**34.8**	**28.4**

	Blood Age		
Endothelial Treatment	**18-36 hrs (n=1)**	**2-16 hrs (n=6)**	**All (n=7)**
Unstimulated SSRBCs, Unstimulated MECs	8.0	24.9	22.5
Unstimulated SSRBCs, TNF-α (6 hrs) on MECs	41.7	48.1	47.2
DETA-NO on SSRBCs, TNF-α (6 hrs) on MECs	41.7	33.7	34.8
% Inhibition	0.0	62.1	50.0

6.5 Chapter Summary

The results presented in Chapter 6 explore how nitric oxide treatment affects sickle erythrocyte adhesion. ELISA assays demonstrate that TNF-α induced VCAM-1 surface expression, but not E-selectin or ICAM-1 expression, is inhibited by pretreatment with and continuous presence of reagents that increase intracellular nitric oxide during TNF-α stimulation. Nitric oxide donors alone (without TNF-α) do not affect VCAM-1, E-selectin, or ICAM-1 surface expression. These biochemical observations translate well to functional observations measuring sickle erythrocyte adhesion. Two different nitric oxide donors inhibit TNF-α induced adhesion by 71% and 79%, presumably by inhibiting VCAM-1 expression. These results are mimicked when TNF-α stimulated MECs are treated with VCAM-1 antibody, which inhibits adhesion by 72% (See Section 4.3.2). Treatment of MECs with NO donors alone (without TNF-α) does not affect sickle erythrocyte adhesion.

Also explored in Chapter 6 is whether treatment of sickle erythrocytes with nitric oxide donors affects their adhesion to endothelial cells. Nitric oxide donors do not affect adhesion to unstimulated endothelial cells.

However, a substantial (28% to 50%), but not statistically significant, inhibition of adhesion to TNF-α stimulated endothelial cells is shown, suggesting that more studies need to be undertaken to establish if and how nitric oxide affects sickle erythrocytes (See Chapter 8).

Together these data demonstrate anti-inflammatory and anti-adhesive properties of nitric oxide. *In vivo*, soluble VCAM-1 is elevated in sickle patients, compared to non-sickle controls, and soluble VCAM-1 levels correlate significantly with endogenous NO bioavailability. That is, patients with lower NO levels show the higher soluble VCAM-1 levels (Gladwin et al., 2003). Furthermore, higher NO metabolite levels in sickle patients experiencing pain episodes correlate with lower pain scores (Lopez *et al.*, 1996). Overall, the data presented in this chapter and elsewhere suggest that the benefits of nitric oxide to sickle cell anemia patients may extend beyond regulation of vascular tone.

CHAPTER 7

CONCLUSIONS

Sickle cell anemia is caused by the homozygous inheritance of the sickle β hemoglobin gene. Symptoms of the conditions include reversible aggregation of hemoglobin inside erythrocytes upon deoxygenation, hemolytic anemia, and vaso-occlusion resulting in painful crises, acute chest syndrome, stroke, retinopathy, priapism, and other complications. In sickle patients, both endothelial cells and erythrocytes exhibit abnormalities that likely contribute to patient clinical symptoms. The sickle endothelium shows dimished capacity to regulate vascular tone and is also chronically activated to express proteins and receptors involved in coagulation and inflammation. One of the most important sickle erythrocyte abnormalities is increased adhesion to endothelial cells and endothelial matrix proteins, which likely initiates or propagates vaso-occlusion. Strategies to inhibit abnormal erythrocyte adhesion to endothelial cells may benefit sickle patients,

but current therapies for sickle cell anemia patients do not directly address adhesion.

The chronic activation of endothelial cells to express and display inflammatory markers may contribute to abnormal sickle erythrocyte adhesion and clinical symptoms. The data presented in the preceding chapters demonstrates that stimulation of endothelial cells with inflammatory cytokines such as TNF-α increases adhesion molecule expression and sickle erythrocyte adhesion *in vitro*; however, expression profiles of adhesion molecules VCAM-1, E-selectin, and ICAM-1 vary with endothelial phenotype. VCAM-1 and E-selectin support adhesion of sickle erythrocytes, whereas ICAM-1 does not. For microvascular endothelial cells, sickle erythrocyte adhesion to unstimulated endothelium is minimal. Stimulation with TNF-α for 2 hrs increases E-selectin, but not VCAM-1 expression, and increases sickle erythrocyte adhesion. Antibody and peptide blocking studies are inconclusive, but suggest that E-selectin may support adhesion of sickle erythrocytes at this stimulation time. Stimulation of MECs with TNF-α for 6 hrs results in significant expression of both VCAM-1 and E-selectin and sickle erythrocyte adhesion is further increased. Antibody and peptide blocking

studies show that both VCAM-1 and E-selectin support adhesion of sickle erythrocytes at this stimulation time.

Together these data show that sickle erythrocyte adhesion is complex *in vitro*, and suggest that adhesion is likely also complex *in vivo*. Dominant mechanisms of adhesion vary with cytokine stimulation time, indicating that strategies to inhibit adhesion *in vivo* may need to encompass multiple adhesive mechanisms to be effective.

Taking this into account, inhibition of sickle erythrocyte adhesion by interruption of the common intracellular signaling cascades initiated by cytokines that lead to expression of endothelial adhesion molecules was studied. Cyclic AMP is a ubiquitous regulator of many intracellular processes and has been shown by others to have many anti-inflammatory properties. The data presented in this work demonstrate that inhibition of endothelial adhesion molecule expression and sickle erythrocyte adhesion is achieved by elevation of endothelial intracellular cAMP levels. The expression of VCAM-1 and E-selectin, but not ICAM-1 on TNF-α stimulated MECs is reduced by treatment of MECs with Forskolin, IBMX, or Bt_2cAMP, which increase endothelial cAMP levels by different mechanisms. The degree of reduction depends primarily on the length of treatment time with cAMP-elevating reagents

and, to a lesser extent, on the concentration of reagent used. While significant reductions in adhesion molecule expression are achieved by pretreatment of MECs with cAMP-elevating reagents, the largest reductions in expression are seen when the reagents are present both prior to and during TNF-α stimulation.

Pretreatment and continuous presence of cAMP-elevating reagents during TNF-α stimulation of MECs also results in significantly decreased sickle erythrocyte adhesion. Adhesion is not, however, completely attenuated, with percent inhibition of adhesion varying from 59% to 64%, depending on the cAMP-elevating reagent used. Incomplete inhibition of sickle erythrocyte adhesion may result from incomplete inhibition of adhesion molecule expression.

Experiments in which sickle erythrocytes, rather than endothelial cells, are treated with cAMP-elevating reagents prior to perfusion show that adhesion to TNF-α stimulated MECs is not altered. However, adhesion to unstimulated MECs is increased for some blood samples, consistent with other reports in the literature.

Like cAMP, nitric oxide also shows potential as an anti-inflammatory molecule as well as a vasodilator. ELISA assays demonstrate that TNF-α induced VCAM-1 surface

expression, but not E-selectin or ICAM-1 expression, is significantly inhibited by pretreatment with and continuous presence of reagents that increase intracellular nitric oxide during TNF-α stimulation. Nitric oxide donors alone (without TNF-α) do not affect VCAM-1, E-selectin, or ICAM-1 surface expression. These biochemical observations translate well to functional observations measuring sickle erythrocyte adhesion. Two different nitric oxide donors inhibit TNF-α induced adhesion by 71% and 79%, presumably by inhibiting VCAM-1 expression. Treatment of MECs with NO donors alone (without TNF-α) does not affect sickle erythrocyte adhesion.

Also explored in this work is whether or not nitric oxide in sickle erythrocytes affects their adhesion to endothelial cells. Elevated nitric oxide does not affect adhesion to unstimulated endothelial cells. However, a substantial (28% to 50%), but not statistically significant, inhibition of adhesion to TNF-α stimulated endothelial cells is shown, suggesting that more studies need to be undertaken to establish if and how nitric oxide affects sickle erythrocytes.

In summary, the data presented in this work suggest that cAMP and nitric oxide and their associated signaling

may significantly affect the pathology of sickle cell anemia. Using an endothelial model in which both VCAM-1 and E-selectin support adhesion of sickle erythrocytes, it is demonstrated that increases in cAMP and NO regulate TNF-α induced expression of one or both receptors and result in reduced sickle erythrocyte adhesion, which may be of benefit to sickle patients. The work suggests that in addition to antibody blocking, interruption of the signaling cascades leading to surface expression of receptors may be effective to control sickle cell adhesion and vaso-occlusion. The effects of cAMP and NO described here, along with those described by others, suggest that cAMP and nitric oxide-dependent signaling may be an important therapeutic target in the prevention or treatment of occlusive events in sickle cell anemia.

CHAPTER 8

RECOMMENDATIONS AND FUTURE STUDIES

8.1 Inhibition of Adhesion Molecule Expression and Sickle Erythrocyte Adhesion at Long Cytokine Stimulation Times

As discussed in Section 5.2.2, treatment of endothelial cells with reagents that increase intracellular cAMP partially, but not completely, inhibits TNF-α induced VCAM-1 and E-selectin expression and sickle erythrocyte adhesion. To be effective for 6 hrs of TNF-α stimulation, the cAMP-elevating reagents must be available for the duration of TNF-α stimulation. This is likely the case because cAMP levels quickly return to baseline after reagents are removed from contact with endothelial cells, presumably because cAMP is broken down to AMP by phosphodieasterases. Also, literature suggests that continuously elevated levels of cAMP in endothelial cells can result in a period of refractoriness, in which

endothelial cells are no longer responsive to cAMP (Krall & Jamgotchian, 1987).

One limitation of the studies presented in the preceding chapters is that they do not address the effects of cAMP-elevating reagents at long cytokine stimulation times. *In vivo*, sickle patients exhibit signs of chronic elevation of cytokine levels which are further elevated during symptomatic events. Thus for a therapy aiming to limit or control the inflammatory process or cytokine-induced cell signaling to be of benefit *in vivo*, it may need to be effective over long periods of time. ELISA or flow cytometry assays should be performed to determine the effect of continous presence of cAMP-elevating reagents on both endothelial cAMP concentrations and cytokine-induced adhesion molecule expression at longer (1-3 days) cytokine stimulation times.

8.2 Inhibition of Leukocyte Rolling and Adhesion by cAMP and NO

Recent reports in literature suggest that in addition to sickle erythrocytes, sickle leukocytes are also abnormally adherent to the endothelium (Kaul et al., 2004; Okpala, 2004). Thus therapies to control adhesion or vaso-

occlusion in sickle cell patients may need to account for both cell types. Since E-selectin on endothelial cells contributes to rolling of leukocytes and cAMP inhibits TNF-α induced E-selectin expression, it is hypothesized that elevated endothelial cAMP inhibits sickle leukocyte rolling and adhesion. This hypothesis could be tested in flow assays similar to those which measure sickle erythrocyte adhesion, except that leukocytes, rather than erythrocytes would be isolated from sickle blood and perfused over treated endothelial cells. In addition to counting adherent leukocytes, numbers and velocities of rolling leukocytes could be measured in the presence and absence of cAMP elevating reagents. Similar experiments could be conducted with NO elevating reagents; however, it is not clear from the data presented in previous chapters that nitric oxide would affect E-selectin expression or leukocyte rolling and adhesion.

8.3 Effect of cAMP and NO on Other Adhesion Molecules Supporting Sickle Erythrocyte Adhesion

The data presented in this document suggest that cAMP and nitric oxide inhibit expression of VCAM-1 and/or E-selectin and sickle erythrocyte adhesion. However, these

are just two of several receptors on endothelial cells shown to support sickle erythrocyte adhesion, and literature indicates that cAMP and nitric oxide may affect surface expression of other receptors important to sickle cell anemia. One study suggests that cAMP alone induces a delayed release of vWF and, to a much lesser extent, P-selectin from Weibel-Palade bodies in endothelial cells (Cleator *et al.*, 2005). Another, however, demonstrates that elevated cAMP actually inhibits histamine-induced P-selectin surface expression (Easton & Dorovini-Zis, 2001). Several *in vivo* studies also suggest that elevated nitric oxide inhibits P-selectin expression (Barbato *et al.*, 2005), while lowering nitric oxide levels induces expression (Nabah *et al.*, 2005).

In addition to endothelial receptors, it is likely that cAMP and nitric oxide also play a role in regulating the ligands on sickle erythrocytes that promote adhesion. In fact, recent research indicates a role for cAMP in regulating expression of ICAM-4 on sickle erythrocytes, which binds to endothelial $\alpha_V\beta_3$ (Zennadi et al., 2004). Also, the results presented in this document suggest that nitric oxide may regulate $\alpha_4\beta_1$ expression (See Section 6.3).

In order to better understand how cAMP or nitric oxide would affect sickle patients *in vivo*, it is necessary to

understand their effects on the adhesion molecules that promote sickle erythrocyte adhesion. ELISA and flow assays could be used to study how these intracellular signaling molecules affect many of the adhesion molecules important to sickle cell anemia patients.

8.4 Regulation of a_4b_1 Expression on Sickle Erythrocytes

The data presented in Section 6.3 suggest that increasing nitric oxide in sickle erythrocytes may inhibit their adhesion to endothelial cells stimulated with inflammatory cytokines, inviting speculation that nitric oxide may regulate sickle erythrocyte $\alpha_4\beta_1$ expression. Little is known about how nitric oxide affects ligands on sickle erythrocytes. However, treatment of neutrophils with reagents that inhibit nitric oxide induces expression of $\alpha_4\beta_1$, which binds to VCAM-1, possibly through mobilization of neutrophil granules and cytoskeletal rearrangement (Conran et al., 2003). It is not clear, though, that NO donors affect $\alpha_4\beta_1$ expression on neutrophils or that the response to NO extends to erythrocytes. In addition, sickle cell patients taking hydroxyurea, which generates NO, show fewer erythrocytes expressing $\alpha_4\beta_1$ and

fewer $\alpha_4\beta_1$ molecules per erythrocyte than sickle patients not taking hydroxyurea (Styles et al., 1997). Although this effect may not result from NO exposure, it invites speculation that one beneficial effect of NO for sickle patients may be reduction in $\alpha_4\beta_1$ expression on sickle erythrocytes and reduced sickle erythrocyte adhesion. To test this hypothesis directly, $\alpha_4\beta_1$ expression on sickle erythrocytes exposed or not to nitric oxide donors could be measured using flow cytometry. In addition, the effect of suppression of nitric oxide synthase enzymes on $\alpha_4\beta_1$ expression and activation could be determined. The determination that nitric oxide affects $\alpha_4\beta_1$ expression would invite further study into the mechanism of action of nitric oxide on sickle erythrocytes.

8.5 Trials of cAMP or Nitric Oxide Elevating Reagents in Sickle Mice

While the studies presented in this document look solely at adhesion molecule expression and sickle erythrocyte adhesion in closely controlled *in vitro* systems, the effects of cAMP and nitric oxide elevating reagents *in vivo* remain to be seen. The development of

sickle transgenic mice allows for study of these two intracellular signaling molecules and their effects on adhesion *in vivo*. To date, many *in vivo* studies in which cAMP or NO elevating reagents are inhaled or injected into sickle mice look only at regulation of vascular tone. Both cAMP-elevating reagents such as forskolin and inhaled nitric oxide appear to improve vascular tone in sickle mice (Kaul et al., 2000). However, it is hypothesized that these reagents may also have anti-inflammatory properties. To test this hypothesis intravital fluorescence microscopy could be used to examine endothelial markers of inflammation such as VCAM-1, E-selectin, ICAM-1, and P-selectin in sickle mice presented with inflammatory challenge or not. The same could be examined after administration of cAMP or nitric oxide elevating reagents. Alternatively, mice could be sacrificed after treatment and tests for endothelial inflammatory markers could be conducted *ex vivo*.

8.6 Effect of cAMP on Endothelial Permeability

Appropriate permeability of endothelial cells is crucial to the regulation of vascular tone and function. The endothelium allows exchange of small molecules such as

water, oxygen, and carbon dioxide between blood and tissues, but prevents exchange of some other, larger molecules. The permeability of endothelial cells varies largely with the type of vessel and the organ system in which they lie. An increase in endothelial permeability due to endothelial damage is a major dysfunction that is part of the common stroke pathogenesis pathway. Thus one hypothesis regarding the pathogenesis of stroke in sickle patients is that sickle erythrocytes damage endothelial cells in such a way as to increase permeability. Lola Brown demonstrates preliminary data suggesting that sickle erythrocytes loosen endothelial adherens junctions, supporting this hypothesis (L. A. Brown, 2005). While much more work is necessary to confirm an effect of sickle erythrocytes on endothelial permeability, confirmation begs the question, “Could prevention of increased endothelial permeability in the sickle vasculature reduce the incidence of stroke in sickle patients, and, if so, how can increased endothelial permeability be prevented?”

Many recent studies indicate that cyclic nucleotides such as cAMP play an important role in the regulation of endothelial permeability. Several *in vitro* assays suggest that cAMP works to tighten endothelial adherens junctions and decrease endothelial permeability. The effect is

independent of endothelial phenotype and has been tested in brain endothelial cells (Bruckener *et al.*, 2003; Chong & Victorino, 2005; Deli *et al.*, 1995; Kooistra *et al.*, 2005; Krizbai & Deli, 2003; Rist *et al.*, 1997). These studies suggest that elevated cAMP, in addition to the effects demonstrated in this document, could potentially reduce the effects of sickle erythrocytes on endothelial permeability. This could be tested in experiments in which endothelial cells grown on polyester membranes are exposed or not to sickle erythrocytes and exposed or not to cAMP-elevating reagents such as Forskolin. Permeability of fluorescently labeled small molecules such as dextran could be measured as previously described (L. A. Brown, 2005).

8.7 Expedition of Endothelial Receptor Shedding

The studies presented in this document have focused on strategies to prevent or inhibit receptor expression and sickle erythrocyte adhesion. However, it is likely that a patient who presents him or herself at a hospital seeking treatment for clinical symptoms associated with sickle cell anemia may already have expressed adhesion molecules, and, thus a preventive strategy may not be effective.

Once receptors are expressed on the endothelial surface, they are displayed for a time, then are shed into the circulation. Another potential treatment strategy, then, may be to expedite the receptor shedding process. An understanding of how receptors are shed is just beginning to develop. It appears that shedding is caused by cleavage of transmembrane proteins by proteases. However, the specific proteases responsible for cleavage of receptors of interest in sickle cell anemia are not well known. Matrix metallo-proteinases are responsible for shedding of most membrane proteins studied to date. Metalloproteinases, possibly the tumor necrosis factor alpha converting enzymes, are responsible for endothelial VCAM-1 shedding (Garton *et al.*, 2003). As more is learned about the enzymes responsible for receptor shedding and the intracellular signaling responsible for their activation, it may be possible to harness their activity for use in treating sickle cell complications.

APPENDIX A

MACROS FOR RECORDING ADHERENCE DATA

Macro to Record Start Time of Flow Adhesion Assay

```
Sub initialtime()
'
' initialtime Macro
' Macro recorded 8/22/2002 by aowings
'
' Keyboard Shortcut: Ctrl+q
'
    ActiveCell.FormulaR1C1 = "=NOW()"
    ActiveCell.Select
    Selection.Copy
    Selection.PasteSpecial Paste:=xlPasteValues,
Operation:=xlNone, SkipBlanks _
        :=False, Transpose:=False
    Application.CutCopyMode = False
    Selection.NumberFormat = "h:mm:ss"
    ActiveCell.Offset(1, 2).Range("A1").Select
End Sub
```

Macro to Record Time of Data Acquisition for Flow Adhesion Assay

```
Sub datetime()
'
' datetime Macro
' Macro recorded 8/22/2002 by aowings
'
' Keyboard Shortcut: Ctrl+s
'
    ActiveCell.Offset(-1, -2).Range("A1").Select
    ActiveCell.FormulaR1C1 = "=NOW()"
    ActiveCell.Select
    Selection.Copy
    Selection.PasteSpecial Paste:=xlPasteValues,
Operation:=xlNone, SkipBlanks _
        :=False, Transpose:=False
    Application.CutCopyMode = False
    Selection.NumberFormat = "h:mm:ss"
    ActiveCell.Offset(0, 1).Range("A1").Select
    ActiveCell.FormulaR1C1 = "=(RC[-1]-R6C1)*1440"
    ActiveCell.Offset(1, 1).Range("A1").Select
End Sub
```

APPENDIX B

KEY DATA

Table B.1 Adhesion Molecule Expression on MECs as a function of TNF-a stimulation time. Average data are presented graphically in Figure 4.1.

VCAM-1	Experiment Number										
TNF-a Stim Time (hrs)	E1	E2	E3	E4	E5	E6	E7	E8	E9	Average	SEM
0	0.065	0.017	0.032	0.030	0.028	0.016	0.006	0.032	0.010	0.026	0.006
2	0.048	0.000	0.024	0.019	0.016	0.007	0.005	0.041	0.024	0.020	0.005
4	0.052	0.033	0.041	0.076	0.019	0.000	0.050	0.096	0.103	0.052	0.011
6	0.051	0.090	0.045	0.187	0.073	0.045	0.186	0.167	0.149	0.110	0.021
8	0.138	0.094	0.064	0.254	0.183	0.010	0.137	0.196	0.228	0.145	0.026

E-selectin	Experiment Number										
TNF-a Stim Time (hrs)	E1	E2	E3	E4	E5	E6	E7	E8	E9	Average	SEM
0	0.000	0.004	0.004	0.000	0.008	0.032	0.048	0.000	0.000	0.011	0.006
2	0.159	0.068	0.056	0.059	0.023	0.021	0.176	0.220	0.352	0.126	0.037
4	0.385	0.281	0.196	0.397	0.199	0.107	0.551	0.433	0.530	0.342	0.052
6	0.448	0.447	0.406	0.504	0.284	0.135	0.597	0.563	0.469	0.428	0.047
8	0.567	0.283	0.437	0.484	0.471	0.301	0.336	0.331	0.508	0.413	0.034

ICAM-1	Experiment Number										
TNF-a Stim Time (hrs)	E1	E2	E3	E4	E5	E6	E7	E8	E9	Average	SEM
0	0.086	0.005	0.037	0.326	0.229	0.173	0.251	0.456	0.112	0.186	0.048
2	0.197	0.129	0.235	0.415	0.265	0.194	0.318	0.727	0.385	0.318	0.060
4	0.411	0.268	0.565	0.732	0.452	0.477	0.597	1.010	0.888	0.600	0.079
6	0.618	0.705	0.990	0.979	0.814	0.775	0.719	1.085	0.965	0.850	0.053
8	0.746	0.725	1.133	1.251	0.993	0.568	0.540	1.103	0.837	0.877	0.085

Table B.2 Sample Calculations for single factor ANOVA and Fisher's Pairwise Comparisons. ELISA data presented in Table B.1 and graphically in Figure 4.1 are used for sample calculations. MINITAB™ Statistical Software is used for all calculations, and software output is shown below. Statistical differences are achieved when ANOVA returns $P<0.05$ and when the intervals returned by Fisher's pairwise comparisons do not contain zero. Statistical differences are shown in bold print for emphasis.

One-way ANOVA: O.D. 450 nm (VCAM-1) versus TNF stimulation time

```
Analysis of Variance for O.D. 450
Source     DF        SS        MS        F        P
TNF stim    4   0.10733   0.02683    11.37    0.000
Error      40   0.09439   0.00236
Total      44   0.20172

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ---+---------+---------+---------+---
0           9   0.02624   0.01751    (----*-----)
2           9   0.02039   0.01614   (----*-----)
4           9   0.05217   0.03437        (-----*----)
6           9   0.11021   0.06157                  (----*-----)
8           9   0.14496   0.07911                       (----*-----)
                                  ---+---------+---------+---------+---
Pooled StDev =  0.04858            0.000     0.060     0.120     0.180

Fisher's pairwise comparisons

    Family error rate = 0.275
Individual error rate = 0.0500

Critical value = 2.021

Intervals for (column level mean) - (row level mean)

                  0          2          4          6

       2    -0.04042
             0.05213

       4    -0.07220   -0.07806
             0.02036    0.01450

       6    -0.13024   -0.13610   -0.10432
            -0.03768   -0.04354   -0.01176

       8    -0.16499   -0.17085   -0.13907   -0.08103
            -0.07243   -0.07829   -0.04651    0.01153
```

One-way ANOVA: O.D. 450 nm (E-selectin) versus TNF stimulation time

```
Analysis of Variance for O.D. 450
Source     DF        SS        MS        F        P
TNF stim    4    1.2608    0.3152    23.37    0.000
Error      40    0.5396    0.0135
Total      44    1.8004

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
0           9    0.0077    0.0197   (---*----)
2           9    0.1259    0.1108         (----*----)
4           9    0.3422    0.1552                (----*----)
6           9    0.4282    0.1423                      (----*----)
8           9    0.4131    0.1023                     (----*----)
                                  -----+---------+---------+---------+-
Pooled StDev =   0.1161              0.00      0.16      0.32      0.48

Fisher's pairwise comparisons

    Family error rate = 0.275
Individual error rate = 0.0500

Critical value = 2.021

Intervals for (column level mean) - (row level mean)

                  0           2           4           6

       2     -0.2289
             -0.0076

       4     -0.4452     -0.3269
             -0.2238     -0.1056

       6     -0.5312     -0.4129     -0.1967
             -0.3099     -0.1916      0.0246

       8     -0.5161     -0.3979     -0.1816     -0.0956
             -0.2948     -0.1766      0.0397      0.1257
```

One-way ANOVA: O.D. 450 nm (ICAM-1) versus TNF stimulation time

```
Analysis of Variance for O.D. 450
Source     DF        SS        MS        F        P
TNF stim    4    3.4570    0.8643    21.55    0.000
Error      40    1.6039    0.0401
Total      44    5.0609

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ---------+---------+---------+-------
0           9    0.1863    0.1454   (---*----)
2           9    0.3183    0.1793       (----*---)
4           9    0.6000    0.2380                (---*---)
6           9    0.8499    0.1597                       (---*----)
8           9    0.8772    0.2551                        (---*----)
                                  ---------+---------+---------+-------
Pooled StDev =   0.2002                 0.30      0.60      0.90
```

Fisher's pairwise comparisons

Family error rate = 0.275
Individual error rate = 0.0500

Critical value = 2.021

Intervals for (column level mean) - (row level mean)

	0	2	4	6
2	-0.3228 0.0588			
4	**-0.6044** **-0.2229**	**-0.4725** **-0.0909**		
6	**-0.8544** **-0.4728**	**-0.7224** **-0.3408**	**-0.4407** **-0.0592**	
8	**-0.8816** **-0.5001**	**-0.7497** **-0.3681**	**-0.4680** **-0.0864**	-0.2181 0.1635

Table B.3 VCAM-1 expression on TNF-a stimulated MECs and HUVECs. Average data are presented graphically in Figure 4.2.

VCAM-1 (MECs)	Experiment Number		
TNF-a Stim Time (hrs)	E4	E5	Average
0	0.030	0.028	0.029
1	0.020	0.014	0.017
2	0.019	0.016	0.017
3	0.026	0.017	0.021
4	0.076	0.019	0.047
6	0.187	0.073	0.130
8	0.254	0.183	0.218
12	0.439	0.334	0.387
16	0.516	0.427	0.472
20	0.464	0.380	0.422
24	0.443	0.368	0.406

VCAM-1 (HUVECs)	Experiment Number		
TNF-a Stim Time (hrs)	E4	E5	Average
0	0.015	0.015	0.015
1	0.005	0.016	0.010
2	0.005	0.010	0.007
3	0.014	0.025	0.020
4	0.036	0.055	0.045
6	0.157	0.184	0.171
8	0.108	0.194	0.151
12	0.212	0.065	0.139
16	0.200	0.136	0.168
20	0.204	0.228	0.216
24	0.226	0.419	0.323

Table B.4 E-selectin expression on TNF-a stimulated MECs and HUVECs. Average data are presented graphically in Figure 4.3.

E-selectin (MECs)	Experiment Number		
TNF-a Stim Time (hrs)	E4	E5	Average
0	0.000	0.008	0.004
1	0.006	0.011	0.009
2	0.059	0.023	0.041
3	0.194	0.089	0.141
4	0.397	0.199	0.298
6	0.504	0.284	0.394
8	0.484	0.471	0.477
12	0.548	0.259	0.404
16	0.500	0.212	0.356
20	0.349	0.119	0.234
24	0.328	0.054	0.191

E-selectin (HUVECs)	Experiment Number		
TNF-a Stim Time (hrs)	E4	E5	Average
0	0.000	0.006	0.003
1	0.009	0.006	0.007
2	0.008	0.036	0.022
3	0.037	0.087	0.062
4	0.062	0.106	0.084
6	0.088	0.214	0.151
8	0.058	0.155	0.107
12	0.098	0.036	0.067
16	0.074	0.049	0.062
20	0.042	0.013	0.028
24	0.042	0.017	0.030

Table B.5 ICAM-1 expression on TNF-a stimulated MECs and HUVECs. Average data are presented graphically in Figure 4.4.

ICAM-1 (MECs)	Experiment Number		
TNF-a Stim Time (hrs)	E4	E5	Average
0	0.326	0.229	0.278
1	0.252	0.212	0.232
2	0.415	0.265	0.340
3	0.576	0.443	0.509
4	0.732	0.452	0.592
6	0.979	0.814	0.896
8	1.251	0.993	1.122
12	1.267	1.175	1.221
16	1.377	1.173	1.275
20	1.455	1.194	1.324
24	1.503	1.184	1.343

ICAM-1 (HUVECs)	Experiment Number		
TNF-a Stim Time (hrs)	E4	E5	Average
0	0.630	0.809	0.719
1	0.598	0.817	0.708
2	0.631	0.768	0.700
3	0.799	0.837	0.818
4	0.781	0.862	0.822
6	0.921	1.025	0.973
8	0.985	1.126	1.056
12	1.041	0.935	0.988
16	1.008	1.029	1.018
20	1.013	1.061	1.037
24	0.883	1.111	0.997

Table B.6 Adhesion of SSRBCs to TNF-a stimulated MECs. Average data are presented graphically in Figure 4.5.

Unstimulated	Experiment Number									
Perfusion Time (min)	S19	S34	S35	S43	S45	S50	S57	S68	Average	SEM
1	3.2	0.0	0.7	2.7	1.1	0.9	0.0	1.9	1.3	0.4
3	5.8	1.8	0.0	9.3	0.0	3.6	4.0	4.3	3.6	1.1
5	2.9	1.6	2.5	5.3	0.9	6.4	16.0	6.4	5.3	1.7
10	3.2	4.2	2.4	11.2	2.1	2.5	15.2	7.2	6.0	1.7
20	0.8	6.7	4.8	11.2	1.5	7.0	22.2	13.2	8.4	2.5
30	5.6	6.4	2.4	16.8	6.5	1.3	19.0	16.8	9.4	2.5
40	2.4	3.3	2.4	23.6	1.5	3.0	14.5	6.2	7.1	2.8

TNF-a (2 hrs)	Experiment Number									
Perfusion Time (min)	S19	S34	S35	S43	S45	S50	S57	S68	Average	SEM
1	0.7	0.0	2.1	1.7	1.7	3.2	5.1	2.4	2.1	0.5
3	1.5	2.9	4.2	7.0	4.0	11.1	8.8	11.2	6.3	1.3
5	4.4	10.7	10.7	8.4	0.7	10.7	17.0	9.9	9.1	1.7
10	6.4	4.6	7.6	9.1	6.5	14.5	16.0	12.4	9.7	1.5
20	15.2	20.2	21.6	24.0	8.0	22.5	18.0	24.7	19.3	2.0
30	22.4	16.0	9.7	19.5	8.8	21.3	17.4	21.1	17.0	1.9
40	12.2	23.6	20.6	21.8	8.4	22.3	16.7	21.3	18.4	1.9

TNF-a (6 hrs)	Experiment Number									
Perfusion Time (min)	S19	S34	S35	S43	S45	S50	S57	S68	Average	SEM
1	4.6	0.0	3.2	2.5	1.7	1.3	5.3	0.7	2.4	0.7
3	6.9	9.5	7.2	10.4	1.7	7.2	8.0	1.3	6.5	1.2
5	20.8	17.7	9.1	14.3	4.8	11.3	12.0	4.7	11.8	2.0
10	35.2	31.2	9.3	30.4	1.5	15.3	10.2	13.2	18.3	4.4
20	52.8	72.8	20.0	52.7	10.9	23.6	20.9	28.8	35.3	7.6
30	57.9	84.2	15.2	68.2	16.0	22.3	26.9	18.5	38.6	9.6
40	40.8	80.8	17.2	68.4	22.1	34.1	30.7	56.9	43.9	8.0

Table B.7 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 2 hrs by anti-VCAM-1 antibody. Average data are presented graphically in Figure 4.6, Panel A.

Unstimulated	Experiment Number									
Perfusion Time (min)	S38	S39	S41	S43	S45	S48	S57	S68	Average	SEM
1	1.5	8.0	0.8	2.7	1.1	8.0	0.0	1.9	3.0	1.1
3	3.2	16.0	2.3	9.3	0.0	0.9	4.0	4.3	5.0	1.9
5	9.1	16.0	2.9	5.3	0.9	5.6	16.0	6.4	7.8	2.0
10	9.7	10.0	2.5	11.2	2.1	12.0	15.2	7.2	8.7	1.6
20	5.3	16.0	1.6	11.2	1.5	9.6	22.2	13.2	10.1	2.5
30	10.0	13.7	2.4	16.8	6.5	10.4	19.0	16.8	12.0	2.0
40	16.0	25.6	8.0	23.6	1.5	15.2	14.5	6.2	13.8	2.9

a-VCAM-1	Experiment Number									
Perfusion Time (min)	S38	S39	S41	S43	S45	S48	S57	S68	Average	SEM
1	1.8	2.7	1.6	1.1	1.7	0.9	1.6	0.8	1.5	0.2
3	18.3	17.6	4.4	4.6	4.2	9.1	8.0	3.3	8.7	2.1
5	18.7	12.8	3.2	8.6	2.5	24.5	3.0	6.3	9.9	2.9
10	23.3	29.5	3.2	10.9	10.7	19.1	7.0	11.3	14.4	3.1
20	34.7	65.8	14.3	9.5	6.1	38.9	12.2	20.7	25.3	7.1
30	35.0	160.0	8.9	16.0	7.6	36.0	13.1	17.8	36.8	18.0
40	28.3	221.7	16.8	23.6	5.3	51.8	9.0	20.8	47.2	25.4

TNF-a (2 hrs)	Experiment Number									
Perfusion Time (min)	S38	S39	S41	S43	S45	S48	S57	S68	Average	SEM
1	2.2	0.0	1.6	1.7	1.7	2.5	5.1	2.4	2.1	0.5
3	5.6	9.6	7.6	7.0	4.0	2.8	8.8	11.2	7.1	1.0
5	13.1	22.9	9.1	8.4	0.7	9.5	17.0	9.9	11.3	2.3
10	30.5	72.0	7.2	9.1	6.5	12.4	16.0	12.4	20.8	7.8
20	23.5	58.7	10.4	24.0	8.0	35.6	18.0	24.7	25.4	5.7
30	31.1	137.6	15.2	19.5	8.8	51.2	17.4	21.1	37.7	15.0
40	33.6	229.3	13.6	21.8	8.4	41.3	16.7	21.3	48.3	26.1

Table B.8 Fold change in sickle erythrocyte adhesion due to 2 hrs of TNF-a stimulation and anti-VCAM-1. Data are presented graphically in Figure 4.6, Panel B.

	Experiment Number							
	S38	**S39**	**S41**	**S43**	**S45**	**S48**	**S57**	**S68**
TNF-a (2 hrs)	2.1	9.0	1.7	0.9	5.8	2.7	1.1	3.5
a-VCAM-1	1.8	8.7	2.1	1.0	3.7	3.4	0.6	3.4

Table B.9 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 2 hrs by E-selectin blocking peptide and anti-VCAM-1 antibody. Average data are presented graphically in Figure 4.7, Panel A.

Unstimulated	Experiment Number									
Perfusion Time (min)	S38	S39	S41	S43	S45	S48	S57	S68	Average	SEM
1	1.5	8.0	0.8	2.7	1.1	8.0	0.0	1.9	3.0	1.1
3	3.2	16.0	2.3	9.3	0.0	0.9	4.0	4.3	5.0	1.9
5	9.1	16.0	2.9	5.3	0.9	5.6	16.0	6.4	7.8	2.0
10	9.7	10.0	2.5	11.2	2.1	12.0	15.2	7.2	8.7	1.6
20	5.3	16.0	1.6	11.2	1.5	9.6	22.2	13.2	10.1	2.5
30	10.0	13.7	2.4	16.8	6.5	10.4	19.0	16.8	12.0	2.0
40	16.0	25.6	8.0	23.6	1.5	15.2	14.5	6.2	13.8	2.9

E-sel pep	Experiment Number									
Perfusion Time (min)	S38	S39	S41	S43	S45	S48	S57	S68	Average	SEM
1	0.8	0.0	2.4	1.9	0.0	2.9	0.0	1.8	1.2	0.4
3	8.6	4.8	5.3	3.2	0.8	10.1	5.7	3.2	5.2	1.1
5	9.8	10.0	3.0	8.5	5.6	8.0	9.8	5.6	7.5	0.9
10	13.5	14.7	0.8	5.6	2.0	23.6	13.9	7.0	10.1	2.7
20	16.8	8.0	3.7	10.7	5.8	117.1	15.2	16.7	24.3	13.4
30	14.2	35.6	7.2	12.4	3.8	116.2	14.0	23.0	28.3	13.0
40	13.1	16.0	4.8	17.6	3.8	155.6	24.0	15.2	31.3	17.9

Data are continued on the following page.

Table B.9, continued.

E-sel pep + ^{a}-VCAM-1	Experiment Number									
Perfusion Time (min)	S38	S39	S41	S43	S45	S48	S57	S68	Average	SEM
1	1.3	1.6	3.8	1.7	0.9	2.4	6.9	1.4	2.5	0.7
3	3.3	4.8	4.8	8.9	3.2	5.0	11.2	7.6	6.1	1.0
5	8.0	6.0	10.4	8.6	7.6	8.0	10.4	8.8	8.5	0.5
10	18.0	8.9	11.2	14.4	2.3	5.1	9.7	6.4	9.5	1.8
20	11.6	25.6	8.8	17.8	3.2	40.0	12.2	11.0	16.3	4.1
30	13.7	29.1	10.4	24.8	4.0	35.2	11.4	12.8	17.7	3.8
40	12.5	18.7	18.5	25.8	5.3	72.4	17.9	20.2	23.9	7.2

TNF-^{a} (2 hrs)	Experiment Number									
Perfusion Time (min)	S38	S39	S41	S43	S45	S48	S57	S68	Average	SEM
1	2.2	0.0	1.6	1.7	1.7	2.5	5.1	2.4	2.1	0.5
3	5.6	9.6	7.6	7.0	4.0	2.8	8.8	11.2	7.1	1.0
5	13.1	22.9	9.1	8.4	0.7	9.5	17.0	9.9	11.3	2.3
10	30.5	72.0	7.2	9.1	6.5	12.4	16.0	12.4	20.8	7.8
20	23.5	58.7	10.4	24.0	8.0	35.6	18.0	24.7	25.4	5.7
30	31.1	137.6	15.2	19.5	8.8	51.2	17.4	21.1	37.7	15.0
40	33.6	229.3	13.6	21.8	8.4	41.3	16.7	21.3	48.3	26.1

Table B.10 Fold change in sickle erythrocyte adhesion due to 2 hrs of TNF-a stimulation and E-selectin blocking peptide. Data are presented graphically in Figure 4.7, Panel B.

	Experiment Number							
	S38	**S39**	**S41**	**S43**	**S45**	**S48**	**S57**	**S68**
TNF-a (2 hrs)	2.1	9.0	1.7	0.9	5.8	2.7	1.1	3.5
E-sel pep	0.8	0.6	0.6	0.7	2.6	10.2	1.7	2.5

Table B.11 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 2 hrs by anti-E-selectin antibody. Average data are presented graphically in Figure 4.8.

Unstimulated	Experiment Number		
Perfusion Time (min)	S26	S68	Average
1	0.8	1.9	1.4
3	6.5	4.3	5.4
5	6.4	6.4	6.4
10	3.2	7.2	5.2
20	8.0	13.2	10.6
30	5.6	16.8	11.2
40	8.0	6.2	7.1

a-E-selectin	Experiment Number		
Perfusion Time (min)	S26	S68	Average
1	0.8	0.9	0.8
3	3.7	2.3	3.0
5	2.4	9.1	5.7
10	11.2	10.7	10.9
20	16.8	17.3	17.0
30	20.8	19.7	20.2
40	21.6	17.3	19.4

TNF-a (2 hrs)	Experiment Number		
Perfusion Time (min)	S26	S68	Average
1	1.8	2.4	2.1
3	8.7	11.2	10.0
5	7.2	9.9	8.6
10	21.6	12.4	17.0
20	28.8	24.7	26.7
30	32.0	21.1	26.6
40	41.6	21.3	31.5

Table B.12 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs by anti-VCAM-1 antibody. Average data are presented graphically in Figure 4.9, Panel A.

Unstimulated	Experiment Number														
Perfusion Time (min)	S40	S42	S43	S45	S46	S47	S53	S54	S55	S60	S63	S65	S68	Average	SEM
1	0.8	1.9	2.7	1.1	4.4	7.7	4.4	4.2	0.0	0.0	0.0	0.0	1.9	2.2	0.7
3	8.0	6.6	9.3	0.0	11.8	6.9	5.3	0.6	2.0	6.5	8.0	3.2	4.3	5.6	1.0
5	6.1	5.3	5.3	0.9	8.0	20.4	7.1	2.6	4.0	4.9	6.2	2.9	6.4	6.2	1.3
10	16.0	9.1	11.2	2.1	5.9	14.3	11.8	0.7	3.0	8.0	21.6	8.0	7.2	9.2	1.6
20	10.9	5.1	11.2	1.5	12.8	21.8	12.0	1.4	4.4	11.9	23.0	4.1	13.2	10.2	1.9
30	22.6	6.4	16.8	6.5	14.4	9.8	18.1	4.2	4.7	7.0	24.5	11.6	16.8	12.6	1.9
40	13.7	7.6	23.6	1.5	15.2	18.3	15.3	0.7	2.8	13.6	26.0	16.6	6.2	12.4	2.3

a-VCAM-1	Experiment Number														
Perfusion Time (min)	S40	S42	S43	S45	S46	S47	S53	S54	S55	S60	S63	S65	S68	Average	SEM
1	3.4	2.3	3.4	1.8	3.2	6.7	3.8	0.6	0.7	2.0	2.3	5.1	1.5	2.8	0.5
3	13.2	5.3	9.6	4.2	8.0	13.2	2.2	4.1	3.3	9.6	9.0	10.7	7.2	7.7	1.0
5	10.4	2.7	15.0	2.7	13.9	14.2	3.1	3.7	7.3	9.8	21.3	14.2	8.0	9.7	1.6
10	30.0	11.2	10.9	3.1	8.0	13.0	6.0	12.5	8.0	7.3	29.7	7.0	9.9	12.0	2.3
20	14.3	16.8	21.3	5.6	7.1	27.8	5.3	17.2	19.2	13.5	41.6	13.1	12.6	16.6	2.7
30	26.1	24.0	26.4	14.4	12.6	18.7	1.5	21.8	32.7	27.6	41.6	21.6	10.1	21.5	2.9
40	21.3	30.6	48.0	8.8	14.5	32.8	17.4	40.7	35.2	25.6	34.8	18.9	19.6	26.8	3.1

TNF-a (6 hrs)	Experiment Number														
Perfusion Time (min)	S40	S42	S43	S45	S46	S47	S53	S54	S55	S60	S63	S65	S68	Average	SEM
1	0.0	6.0	2.5	1.7	12.8	1.9	7.0	4.2	3.4	3.6	1.5	1.5	0.7	3.6	0.9
3	4.6	9.8	10.4	1.7	45.1	6.2	14.1	2.2	8.0	12.7	30.7	9.3	1.3	12.0	3.5
5	13.7	22.4	14.3	4.8	46.7	28.4	23.5	5.6	9.6	10.0	27.4	8.5	4.7	16.9	3.4
10	36.6	19.6	30.4	1.5	53.1	28.5	29.2	17.2	16.0	33.3	69.0	20.4	13.2	28.3	4.9
20	41.0	56.8	52.7	10.9	88.9	48.8	36.7	24.3	19.6	39.0	104.5	32.0	28.8	44.9	7.4
30	46.2	54.1	68.2	16.0	119.1	54.5	29.2	46.1	32.0	33.9	140.4	51.0	18.5	54.6	10.2
40	35.8	49.8	68.4	22.1	137.1	46.3	63.3	42.4	29.0	53.6	160.0	54.7	56.9	63.0	11.2

Table B.13 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs by an E-selectin blocking peptide and/or anti-VCAM-1 antibody. Average data are presented graphically in Figure 4.9, Panel B.

Unstimulated	Experiment Number										
Perfusion Time (min)	S40	S42	S43	S45	S46	S47	S63	S65	S68	Average	SEM
1	0.8	1.9	2.7	1.1	4.4	7.7	0.0	0.0	1.9	2.3	0.8
3	8.0	6.6	9.3	0.0	11.8	6.9	8.0	3.2	4.3	6.4	1.2
5	6.1	5.3	5.3	0.9	8.0	20.4	6.2	2.9	6.4	6.8	1.8
10	16.0	9.1	11.2	2.1	5.9	14.3	21.6	8.0	7.2	10.6	2.0
20	10.9	5.1	11.2	1.5	12.8	21.8	23.0	4.1	13.2	11.5	2.5
30	22.6	6.4	16.8	6.5	14.4	9.8	24.5	11.6	16.8	14.4	2.2
40	13.7	7.6	23.6	1.5	15.2	18.3	26.0	16.6	6.2	14.3	2.7

E-sel pep	Experiment Number										
Perfusion Time (min)	S40	S42	S43	S45	S46	S47	S63	S65	S68	Average	SEM
1	2.5	6.0	1.8	3.4	5.6	5.1	7.5	0.0	0.0	3.5	0.9
3	11.4	24.0	10.4	4.4	10.7	21.6	10.7	6.2	6.5	11.8	2.2
5	22.2	10.7	12.8	4.8	14.5	11.3	37.7	10.1	16.0	15.6	3.2
10	30.1	26.7	16.0	4.9	22.9	29.1	28.8	11.5	10.7	20.1	3.2
20	26.9	36.6	30.8	8.0	19.8	34.4	46.0	12.5	9.8	25.0	4.4
30	39.1	57.9	48.0	7.3	32.7	34.8	55.0	18.8	16.0	34.4	5.9
40	40.5	64.0	56.4	7.6	31.3	36.4	62.9	21.1	15.2	37.3	6.9

Data are continued on the following page.

Table B.13, continued.

E-sel pep + ^{a}-VCAM-1	Experiment Number										
Perfusion Time (min)	S40	S42	S43	S45	S46	S47	S63	S65	S68	Average	SEM
1	1.3	1.1	3.2	7.0	4.0	4.9	4.0	0.8	2.0	3.1	0.7
3	8.0	7.0	11.4	5.8	2.4	14.4	3.4	7.6	6.5	7.4	1.2
5	6.9	8.0	11.4	5.1	15.0	8.5	12.8	13.2	7.0	9.8	1.1
10	8.3	12.0	10.7	6.9	17.4	18.4	16.0	17.3	14.7	13.5	1.4
20	21.3	20.8	18.2	2.3	32.8	19.2	31.2	18.5	12.7	19.7	3.0
30	24.9	29.6	26.7	5.1	44.5	10.1	45.9	32.0	14.5	25.9	4.7
40	24.0	17.1	32.0	8.8	44.2	24.7	31.2	20.0	15.2	24.1	3.5

TNF-^{a} (6 hrs)	Experiment Number										
Perfusion Time (min)	S40	S42	S43	S45	S46	S47	S63	S65	S68	Average	SEM
1	0.0	6.0	2.5	1.7	12.8	1.9	1.5	1.5	0.7	3.2	1.3
3	4.6	9.8	10.4	1.7	45.1	6.2	30.7	9.3	1.3	13.2	4.9
5	13.7	22.4	14.3	4.8	46.7	28.4	27.4	8.5	4.7	19.0	4.6
10	36.6	19.6	30.4	1.5	53.1	28.5	69.0	20.4	13.2	30.2	6.9
20	41.0	56.8	52.7	10.9	88.9	48.8	104.5	32.0	28.8	51.6	9.8
30	46.2	54.1	68.2	16.0	119.1	54.5	140.4	51.0	18.5	63.1	13.9
40	35.8	49.8	68.4	22.1	137.1	46.3	160.0	54.7	56.9	70.1	15.6

Table B.14 Blocking of sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs by anti-E-selectin antibody. Average data are presented graphically in Figure 4.9, Panel C.

Unstimulated	Experiment Number											
Perfusion Time (min)	S53	S54	S55	S58	S60	S62	S63	S65	S66	S68	Average	SEM
1	4.4	4.2	0.0	0.8	0.0	2.5	0.0	0.0	0.8	1.9	1.5	0.5
3	5.3	0.6	2.0	0.8	6.5	3.4	8.0	3.2	4.4	4.3	3.9	0.7
5	7.1	2.6	4.0	3.0	4.9	4.8	6.2	2.9	9.3	6.4	5.1	0.7
10	11.8	0.7	3.0	3.6	8.0	8.0	21.6	8.0	11.1	7.2	8.3	1.8
20	12.0	1.4	4.4	9.9	11.9	9.6	23.0	4.1	11.1	13.2	10.1	1.9
30	18.1	4.2	4.7	8.3	7.0	5.1	24.5	11.6	12.5	16.8	11.3	2.1
40	15.3	0.7	2.8	6.4	13.6	16.0	26.0	16.6	14.2	6.2	11.8	2.4

a-E-selectin	Experiment Number											
Perfusion Time (min)	S53	S54	S55	S58	S60	S62	S63	S65	S66	S68	Average	SEM
1	1.5	0.7	2.2	1.1	4.0	0.0	0.0	3.0	2.7	0.7	1.6	0.4
3	7.3	0.0	3.8	3.0	3.0	1.7	6.2	3.8	2.0	5.3	3.6	0.7
5	4.0	2.2	5.0	4.9	3.6	4.6	7.5	12.4	6.2	9.5	6.0	1.0
10	15.3	10.4	16.0	6.0	4.7	11.1	8.5	15.4	13.5	13.2	11.4	1.3
20	21.1	18.1	28.2	7.0	6.3	11.8	19.0	20.2	16.0	15.3	16.3	2.1
30	30.2	26.4	45.1	9.9	9.8	16.0	31.0	28.4	24.0	11.1	23.2	3.6
40	35.5	32.0	54.7	10.5	6.7	14.8	33.9	30.5	14.3	21.6	25.4	4.6

TNF-a (6 hrs)	Experiment Number											
Perfusion Time (min)	S53	S54	S55	S58	S60	S62	S63	S65	S66	S68	Average	SEM
1	7.0	4.2	3.4	1.5	3.6	0.7	1.5	1.5	2.2	0.7	2.6	0.6
3	14.1	2.2	8.0	6.0	12.7	4.7	30.7	9.3	2.2	1.3	9.1	2.8
5	23.5	5.6	9.6	7.4	10.0	5.9	27.4	8.5	7.6	4.7	11.0	2.5
10	29.2	17.2	16.0	12.5	33.3	14.2	69.0	20.4	17.4	13.2	24.2	5.4
20	36.7	24.3	19.6	13.7	39.0	9.7	104.5	32.0	35.2	28.8	34.4	8.4
30	29.2	46.1	32.0	14.5	33.9	23.3	140.4	51.0	28.2	18.5	41.7	11.5
40	63.3	42.4	29.0	17.4	53.6	29.1	160.0	54.7	24.5	56.9	53.1	12.9

Table B.15 Sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs and treated with anti-ICAM-1. Average data are presented graphically in Figure 4.10, Panel A.

Unstimulated	Experiment Number					
Perfusion Time (min)	S40	S42	S47	S48	Average	SEM
1	0.8	1.9	7.7	3.8	3.5	1.5
3	8.0	6.6	6.9	5.3	6.7	0.5
5	6.1	5.3	20.4	5.1	9.2	3.7
10	16.0	9.1	14.3	5.9	11.3	2.3
20	10.9	5.1	21.8	6.4	11.0	3.8
30	22.6	6.4	9.8	10.4	12.3	3.5
40	13.7	7.6	18.3	6.4	11.5	2.8

a-ICAM-1	Experiment Number					
Perfusion Time (min)	S40	S42	S47	S48	Average	SEM
1	2.8	0.0	4.8	5.6	3.3	1.2
3	7.5	7.6	28.2	9.4	13.2	5.0
5	6.4	12.2	22.4	36.7	19.4	6.6
10	16.7	13.9	19.2	36.9	21.7	5.2
20	40.5	26.9	29.8	60.8	39.5	7.7
30	49.1	64.8	40.9	80.8	58.9	8.8
40	62.7	51.4	39.6	112.0	66.4	15.9

TNF-a (6 hrs)	Experiment Number					
Perfusion Time (min)	S40	S42	S47	S48	Average	SEM
1	0.0	6.0	1.9	1.1	2.2	1.3
3	4.6	9.8	6.2	0.0	5.1	2.0
5	13.7	22.4	28.4	14.2	19.7	3.5
10	36.6	19.6	28.5	32.8	29.4	3.7
20	41.0	56.8	48.8	37.6	46.1	4.3
30	46.2	54.1	54.5	66.4	55.3	4.2
40	35.8	49.8	46.3	108.0	60.0	16.3

Table B.16 Sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs and treated with anti-IgGk antibody. Average data are presented graphically in Figure 4.10, Panel B.

Unstimulated	Experiment Number										
Perfusion Time (min)	S53	S54	S55	S60	S62	S63	S65	S66	S68	Average	SEM
1	4.4	4.2	0.0	0.0	2.5	0.0	0.0	0.8	1.9	1.5	0.6
3	5.3	0.6	2.0	6.5	3.4	8.0	3.2	4.4	4.3	4.2	0.7
5	7.1	2.6	4.0	4.9	4.8	6.2	2.9	9.3	6.4	5.3	0.7
10	11.8	0.7	3.0	8.0	8.0	21.6	8.0	11.1	7.2	8.8	2.0
20	12.0	1.4	4.4	11.9	9.6	23.0	4.1	11.1	13.2	10.1	2.1
30	18.1	4.2	4.7	7.0	5.1	24.5	11.6	12.5	16.8	11.6	2.4
40	15.3	0.7	2.8	13.6	16.0	26.0	16.6	14.2	6.2	12.4	2.6

a- IgG?	Experiment Number										
Perfusion Time (min)	S53	S54	S55	S60	S62	S63	S65	S66	S68	Average	SEM
1	2.4	3.0	0.9	3.1	2.0	4.4	0.0	1.7	1.2	2.1	0.4
3	7.7	1.5	7.5	5.8	4.3	9.6	2.4	9.6	3.3	5.7	1.0
5	12.2	4.3	12.0	17.5	11.1	18.1	9.3	11.3	4.7	11.2	1.6
10	28.8	11.1	28.2	15.3	17.9	30.2	24.3	20.2	13.3	21.1	2.4
20	42.2	19.3	41.3	36.7	33.3	48.0	24.9	22.3	19.2	31.9	3.6
30	46.7	31.3	64.0	46.9	78.1	64.0	48.0	27.5	26.7	48.1	6.0
40	45.0	33.5	49.7	42.3	49.9	70.6	39.0	42.2	33.9	45.1	3.7

TNF-a (6 hrs)	Experiment Number										
Perfusion Time (min)	S53	S54	S55	S60	S62	S63	S65	S66	S68	Average	SEM
1	7.0	4.2	3.4	3.6	0.7	1.5	1.5	2.2	0.7	2.8	0.7
3	14.1	2.2	8.0	12.7	4.7	30.7	9.3	2.2	1.3	9.5	3.1
5	23.5	5.6	9.6	10.0	5.9	27.4	8.5	7.6	4.7	11.4	2.7
10	29.2	17.2	16.0	33.3	14.2	69.0	20.4	17.4	13.2	25.5	5.9
20	36.7	24.3	19.6	39.0	9.7	104.5	32.0	35.2	28.8	36.7	9.0
30	29.2	46.1	32.0	33.9	23.3	140.4	51.0	28.2	18.5	44.8	12.4
40	63.3	42.4	29.0	53.6	29.1	160.0	54.7	24.5	56.9	57.1	13.7

Table B.17 Sickle erythrocyte adhesion when erythrocytes were incubated with E-selectin blocking peptide, centrifuged, and resuspended in peptide-free media prior to perfusion. Average data are presented graphically in Figure 4.11, Panel A.

Unstimulated	Experiment Number				
Perfusion Time (min)	S42	S60	S62	Average	SEM
1	1.9	0.0	2.5	1.4	0.7
3	6.6	6.5	3.4	5.5	1.0
5	5.3	4.9	4.8	5.0	0.2
10	9.1	8.0	8.0	8.4	0.4
20	5.1	11.9	9.6	8.8	2.0
30	6.4	7.0	5.1	6.2	0.5
40	7.6	13.6	16.0	12.4	2.5

E-sel pep washed away	Experiment Number				
Perfusion Time (min)	S42	S60	S62	Average	SEM
1	2.5	1.0	2.7	2.1	0.5
3	9.6	10.0	0.7	6.8	3.0
5	33.6	3.0	3.1	13.2	10.2
10	32.0	9.6	6.8	16.1	8.0
20	65.9	18.1	10.9	31.6	17.3
30	94.3	18.1	20.0	44.1	25.1
40	78.4	12.6	22.0	37.7	20.5

TNF-^{a} (6 hrs)	Experiment Number				
Perfusion Time (min)	S42	S60	S62	Average	SEM
1	6.0	3.6	0.7	3.5	1.5
3	9.8	12.7	4.7	9.1	2.3
5	22.4	10.0	5.9	12.8	5.0
10	19.6	33.3	14.2	22.3	5.7
20	56.8	39.0	9.7	35.2	13.7
30	54.1	33.9	23.3	37.1	9.0
40	49.8	53.6	29.1	44.2	7.6

Table B.18 Sickle erythrocyte adhesion to MECs stimulated with TNF-a for 6 hrs and treated with scrambled peptide. Average data are presented graphically in Figure 4.11, Panel B.

Unstimulated	Experiment Number				
Perfusion Time (min)	S40	S42	S47	Average	SEM
1	0.8	1.9	7.7	3.5	2.1
3	8.0	6.6	6.9	7.1	0.4
5	6.1	5.3	20.4	10.6	4.9
10	16.0	9.1	14.3	13.2	2.1
20	10.9	5.1	21.8	12.6	4.9
30	22.6	6.4	9.8	12.9	4.9
40	13.7	7.6	18.3	13.2	3.1

Scrambled pep	Experiment Number				
Perfusion Time (min)	S40	S42	S47	Average	SEM
1	4.2	1.9	6.3	4.1	1.3
3	15.0	5.9	16.0	12.3	3.2
5	25.8	8.0	23.1	19.0	5.5
10	41.8	28.0	24.8	31.5	5.2
20	32.7	35.8	33.7	34.1	0.9
30	50.5	59.4	26.9	45.6	9.7
40	54.6	61.7	32.0	49.4	9.0

TNF-a (6 hrs)	Experiment Number				
Perfusion Time (min)	S40	S42	S47	Average	SEM
1	0.0	6.0	1.9	2.6	1.8
3	4.6	9.8	6.2	6.9	1.6
5	13.7	22.4	28.4	21.5	4.3
10	36.6	19.6	28.5	28.2	4.9
20	41.0	56.8	48.8	48.9	4.6
30	46.2	54.1	54.5	51.6	2.7
40	35.8	49.8	46.3	44.0	4.2

Table B.19 Adhesion of sickle erythrocytes treated with anti-alpha 4 antibody to MECs stimulated with TNF-a for 6 hrs. Average data are presented graphically in Figure 4.12, Panel A.

Unstimulated	Experiment Number						
Perfusion Time (min)	S53	S54	S55	S58	S65	Average	SEM
1	4.4	4.2	0.0	0.8	0.0	1.9	1.0
3	5.3	0.6	2.0	0.8	3.2	2.4	0.9
5	7.1	2.6	4.0	3.0	2.9	3.9	0.8
10	11.8	0.7	3.0	3.6	8.0	5.4	2.0
20	12.0	1.4	4.4	9.9	4.1	6.4	2.0
30	18.1	4.2	4.7	8.3	11.6	9.4	2.6
40	15.3	0.7	2.8	6.4	16.6	8.4	3.2

a_4	Experiment Number						
Perfusion Time (min)	S53	S54	S55	S58	S65	Average	SEM
1	0.0	0.6	0.8	0.0	3.4	0.9	0.6
3	6.9	4.3	3.8	4.2	5.1	4.8	0.5
5	10.7	2.1	5.5	9.6	12.2	8.0	1.8
10	17.5	8.3	9.5	8.3	19.0	12.5	2.3
20	36.4	12.5	16.0	16.0	32.9	22.8	4.9
30	41.0	13.9	18.9	33.5	84.6	38.4	12.5
40	45.7	10.9	25.8	46.4	65.6	38.9	9.4

TNF-a (6 hrs)	Experiment Number						
Perfusion Time (min)	S53	S54	S55	S58	S65	Average	SEM
1	7.0	4.2	3.4	1.5	1.5	3.5	1.0
3	14.1	2.2	8.0	6.0	9.3	7.9	2.0
5	23.5	5.6	9.6	7.4	8.5	10.9	3.2
10	29.2	17.2	16.0	12.5	20.4	19.1	2.8
20	36.7	24.3	19.6	13.7	32.0	25.3	4.1
30	29.2	46.1	32.0	14.5	51.0	34.6	6.5
40	63.3	42.4	29.0	17.4	54.7	41.4	8.3

Table B.20 Adhesion of sickle erythrocytes treated with neuraminidase to MECs stimulated with TNF-a for 6 hrs. Average data are presented graphically in Figure 4.12, Panel B.

Unstimulated	Experiment Number					
Perfusion Time (min)	S53	S54	S55	S65	Average	SEM
1	4.4	4.2	0.0	0.0	2.2	1.3
3	5.3	0.6	2.0	3.2	2.8	1.0
5	7.1	2.6	4.0	2.9	4.1	1.0
10	11.8	0.7	3.0	8.0	5.9	2.5
20	12.0	1.4	4.4	4.1	5.5	2.3
30	18.1	4.2	4.7	11.6	9.6	3.3
40	15.3	0.7	2.8	16.6	8.8	4.1

Neuraminidase	Experiment Number					
Perfusion Time (min)	S53	S54	S55	S65	Average	SEM
1	6.0	0.7	0.9	1.8	2.4	1.2
3	6.7	5.8	6.0	13.3	7.9	1.8
5	16.6	6.9	14.6	25.1	15.8	3.8
10	20.2	15.3	21.6	36.0	23.3	4.4
20	32.8	37.9	40.0	40.6	37.8	1.8
30	32.0	38.8	47.3	55.4	43.4	5.1
40	41.5	46.6	52.4	32.0	43.1	4.3

TNF-a (6 hrs)	Experiment Number					
Perfusion Time (min)	S53	S54	S55	S65	Average	SEM
1	7.0	4.2	3.4	1.5	4.0	1.1
3	14.1	2.2	8.0	9.3	8.4	2.5
5	23.5	5.6	9.6	8.5	11.8	4.0
10	29.2	17.2	16.0	20.4	20.7	3.0
20	36.7	24.3	19.6	32.0	28.2	3.8
30	29.2	46.1	32.0	51.0	39.6	5.3
40	63.3	42.4	29.0	54.7	47.3	7.5

Table B.21 Effect of forskolin pretreatment concentration on TNF-a induced adhesion molecule expression. Average data are presented graphically in Figure 5.1.

VCAM-1	Experiment Number							
Forskolin Conc. (uM)	E4	E5	E6	E10	E11	E12	Average	SEM
0 (No TNF-a)	0.094	0.017	0.032	0.000	0.036	0.036	0.036	0.013
0	0.051	0.090	0.045	0.263	0.399	0.399	0.208	0.069
10	0.076	0.063	0.017	0.185	0.380	0.380	0.183	0.066
50	0.100	0.064	0.036	0.108	0.319	0.319	0.158	0.052
100	0.103	0.059	0.042	0.114	0.314	0.314	0.158	0.051
500	0.018	0.031	0.037	0.159	0.248	0.248	0.124	0.044
1000	0.015	0.055	0.013	0.000	0.139	0.139	0.060	0.026

E-selectin	Experiment Number							
Forskolin Conc. (uM)	E4	E5	E6	E10	E11	E12	Average	SEM
0 (No TNF-a)	0.000	0.004	0.004	0.000	0.022	0.022	0.009	0.004
0	0.448	0.447	0.406	0.443	0.507	0.507	0.460	0.016
10	0.423	0.307	0.255	0.332	0.419	0.419	0.359	0.029
50	0.346	0.317	0.314	0.314	0.501	0.501	0.382	0.038
100	0.384	0.212	0.288	0.378	0.410	0.410	0.347	0.033
500	0.295	0.163	0.200	0.332	0.292	0.292	0.262	0.027
1000	0.162	0.049	0.131	0.151	0.237	0.237	0.161	0.029

ICAM-1	Experiment Number							
Forskolin Conc. (uM)	E4	E5	E6	E10	E11	E12	Average	SEM
0 (No TNF-a)	0.086	0.005	0.037	0.018	0.230	0.230	0.101	0.042
0	0.618	0.705	0.990	0.868	0.969	0.969	0.853	0.064
10	0.511	0.488	0.867	0.748	0.856	0.856	0.721	0.072
50	0.612	0.530	0.883	0.692	0.938	0.938	0.765	0.072
100	0.585	0.507	0.836	0.681	0.959	0.959	0.755	0.079
500	0.380	0.431	0.771	0.741	0.851	0.851	0.671	0.086
1000	0.279	0.357	0.637	0.567	0.815	0.815	0.578	0.092

Table B.22 Effect of forskolin treatment time on TNF-a induced adhesion molecule expression. Average data are presented graphically in Figure 5.2.

VCAM-1	Experiment Number					
Endothelial Treatment	E8	E10	E11	E12	Average	SEM
Unstimulated	0.000	0.019	0.071	0.071	0.040	0.018
TNF-a (6 hrs)	0.184	0.217	0.366	0.366	0.283	0.048
Fsk (15 min pre)	0.113	0.186	0.191	0.191	0.170	0.019
Fsk (30 min pre)	0.098	0.223	0.334	0.334	0.247	0.056
Fsk (60 min pre)	0.084	0.202	0.220	0.220	0.181	0.033
Fsk (90 min pre)	0.057	0.147	0.221	0.221	0.161	0.039
Fsk (120 min pre)	0.090	0.188	0.199	0.199	0.169	0.027
Fsk (30 min pre + cont)	0.024	0.032	0.040	0.040	0.034	0.004
Fsk (120 min pre + cont)	0.030	0.042	0.074	0.074	0.055	0.011

E-selectin	Experiment Number					
Endothelial Treatment	E8	E10	E11	E12	Average	SEM
Unstimulated	0.000	0.003	0.007	0.007	0.004	0.002
TNF-a (6 hrs)	0.536	0.418	0.489	0.489	0.483	0.024
Fsk (15 min pre)	0.334	0.264	0.339	0.339	0.319	0.018
Fsk (30 min pre)	0.380	0.366	0.368	0.368	0.370	0.003
Fsk (60 min pre)	0.383	0.299	0.315	0.315	0.328	0.019
Fsk (90 min pre)	0.267	0.308	0.382	0.382	0.335	0.029
Fsk (120 min pre)	0.380	0.338	0.402	0.402	0.381	0.015
Fsk (30 min pre + cont)	0.153	0.134	0.210	0.210	0.177	0.020
Fsk (120 min pre + cont)	0.114	0.146	0.286	0.286	0.208	0.046

ICAM-1	Experiment Number					
Endothelial Treatment	E8	E10	E11	E12	Average	SEM
Unstimulated	0.231	0.124	0.222	0.222	0.200	0.025
TNF-a (6 hrs)	0.875	0.887	0.978	0.978	0.930	0.028
Fsk (15 min pre)	0.631	0.793	0.987	0.987	0.850	0.086
Fsk (30 min pre)	0.762	0.750	0.933	0.933	0.845	0.051
Fsk (60 min pre)	0.690	0.699	0.933	0.933	0.814	0.069
Fsk (90 min pre)	0.562	0.777	0.972	0.972	0.821	0.098
Fsk (120 min pre)	0.710	0.796	0.934	0.934	0.843	0.055
Fsk (30 min pre + cont)	0.533	0.606	0.885	0.885	0.727	0.092
Fsk (120 min pre + cont)	0.518	0.705	0.952	0.952	0.782	0.105

Table B.23 Effect of IBMX concentration on TNF-a induced adhesion molecule expression. Average data are presented graphically in Figure 5.3.

VCAM-1	Experiment Number						
IBMX Concentration (μM)	E20	E21	E22	E23	E24	Average	SEM
0 (No TNF-a)	0.016	0.010	0.003	0.011	0.013	0.010	0.002
0	0.045	0.216	0.023	0.163	0.175	0.124	0.041
10	0.018	0.115	0.057	0.105	0.092	0.077	0.020
50	0.030	0.087	0.060	0.106	0.083	0.073	0.015
100	0.029	0.086	0.040	0.039	0.081	0.055	0.011
500	0.013	0.033	0.023	0.014	0.030	0.023	0.004
1000	0.004	0.006	0.000	0.009	0.002	0.004	0.002

E-selectin	Experiment Number						
IBMX Concentration (μM)	E20	E21	E22	E23	E24	Average	SEM
0 (No TNF-a)	0.032	0.000	0.005	0.000	0.017	0.011	0.007
0	0.135	0.402	0.352	0.367	0.399	0.331	0.054
10	0.217	0.443	0.482	0.350	0.327	0.364	0.053
50	0.205	0.396	0.282	0.304	0.195	0.276	0.035
100	0.164	0.321	0.377	0.108	0.351	0.264	0.057
500	0.182	0.187	0.382	0.220	0.317	0.258	0.042
1000	0.102	0.180	0.338	0.137	0.193	0.190	0.047

ICAM-1	Experiment Number						
IBMX Concentration (μM)	E20	E21	E22	E23	E24	Average	SEM
0 (No TNF-a)	0.173	0.186	0.202	0.621	0.200	0.277	0.097
0	0.775	1.122	0.418	1.244	0.660	0.844	0.166
10	0.787	1.174	0.702	1.088	0.547	0.859	0.102
50	0.784	1.208	0.563	1.074	0.675	0.861	0.130
100	0.664	1.013	0.618	0.780	0.571	0.729	0.079
500	0.628	0.766	0.657	0.907	0.617	0.715	0.056
1000	0.504	0.665	0.651	0.968	0.537	0.665	0.087

Table B.24 Effect of Bt_2cAMP concentration on TNF-a induced adhesion molecule expression. Average data are presented graphically in Figure 5.4.

VCAM-1	Experiment Number						
Bt_2cAMP Concentration (μM)	E20	E21	E22	E23	E24	Average	SEM
0 (No TNF-a)	0.014	0.000	0.006	0.032	0.010	0.012	0.006
0	0.038	0.104	0.186	0.167	0.149	0.129	0.030
10	0.023	0.073	0.156	0.153	0.122	0.105	0.029
50	0.015	0.050	0.074	0.152	0.118	0.082	0.026
100	0.022	0.049	0.005	0.115	0.079	0.054	0.022
500	0.000	0.045	0.026	0.136	0.100	0.061	0.026
1000	0.001	0.032	0.073	0.088	0.058	0.050	0.018

E-selectin	Experiment Number						
Bt_2cAMP Concentration (μM)	E20	E21	E22	E23	E24	Average	SEM
0 (No TNF-a)	0.013	0.000	0.006	0.000	0.000	0.004	0.003
0	0.228	0.552	0.597	0.563	0.469	0.482	0.077
10	0.288	0.539	0.675	0.458	0.447	0.481	0.072
50	0.464	0.291	0.490	0.518	0.395	0.432	0.046
100	0.202	0.392	0.298	0.566	0.011	0.294	0.069
500	0.162	0.370	0.298	0.441	0.314	0.317	0.053
1000	0.080	0.447	0.284	0.399	0.315	0.305	0.073

ICAM-1	Experiment Number
Bt_2cAMP Concentration (μM)	E21
0 (No TNF-a)	1.233
0	1.071
10	0.941
50	1.077
100	1.021
500	0.997
1000	0.195

Table B.25 Comparison of the effect of forskolin on MECs stimulated with TNF-a for 2 or 6 hrs. Data are presented graphically in Figure 5.5.

Endothelial Treatment	VCAM-1	E-selectin	ICAM-1	VCAM-1 SEM	E-selectin SEM	ICAM-1 SEM
Unstimulated	0.040	0.004	0.200	0.018	0.002	0.025
TNF-a (6 hrs)	0.283	0.483	0.930	0.048	0.024	0.028
Fsk (pre) + TNF-a (6 hrs)	0.247	0.370	0.845	0.056	0.003	0.051
Fsk (pre+cont) + TNF-a (6 hrs)	0.034	0.177	0.727	0.004	0.020	0.092

Endothelial Treatment	VCAM-1	E-selectin	ICAM-1	VCAM-1 SEM	E-selectin SEM	ICAM-1 SEM
Unstimulated	0.029	0.005	0.157	0.014	0.002	0.084
TNF-a (2 hrs)	0.012	0.119	0.304	0.005	0.044	0.102
Fsk (pre) + TNF-a (2 hrs)	0.011	0.049	0.259	0.007	0.013	0.088
Fsk (pre+cont) + TNF-a (2 hrs)	0.013	0.010	0.225	0.005	0.003	0.092

Table B.26 Effect of Fsk, IBMX, or Bt_2cAMP alone on adhesion molecule expression. Average data are presented in Table 5.1.

VCAM-1	Experiment Number		
Endothelial Treatment	**E30**	**E31**	**Average**
Unstimulated	0.026	0.011	0.019
Fsk	0.026	0.026	0.018
IBMX	0.038	0.038	0.032
Bt_2cAMP	0.035	0.035	0.026

E-selectin	Experiment Number		
Endothelial Treatment	**E30**	**E31**	**Average**
Unstimulated	0.030	0.036	0.033
Fsk	0.041	0.012	0.027
IBMX	0.046	0.025	0.035
Bt_2cAMP	0.086	0.051	0.069

ICAM-1	Experiment Number		
Endothelial Treatment	**E30**	**E31**	**Average**
Unstimulated	0.123	0.145	0.134
Fsk	0.159	0.098	0.129
IBMX	0.103	0.106	0.104
Bt_2cAMP	0.140	0.114	0.127

Table B.27 Effect of 100 µM forskolin pretreatment on sickle erythrocyte adhesion induced by 2 hrs TNF-a stimulation. Average data are presented graphically in Figure 5.6.

Unstimulated	Experiment Number						
Perfusion Time (min)	S19	S22	S26	S33	S69	Average	SEM
1	3.2	0.0	0.8	1.8	0.0	1.2	0.6
3	5.8	0.0	6.5	5.3	1.2	3.8	1.3
5	2.9	5.3	6.4	5.3	2.7	4.5	0.7
10	3.2	5.6	3.2	3.0	4.4	3.9	0.5
20	0.8	9.6	8.0	10.9	8.0	7.5	1.8
30	5.6	6.4	5.6	7.6	4.4	5.9	0.5
40	2.4	9.6	8.0	6.9	6.7	6.7	1.2

Fsk (pre) + TNF-a (2 hrs)	Experiment Number						
Perfusion Time (min)	S19	S22	S26	S33	S69	Average	SEM
1	3.0	0.0	2.4	0.0	0.0	1.1	0.7
3	4.0	0.7	0.8	11.6	6.4	4.7	2.0
5	1.8	2.0	6.1	8.0	12.0	6.0	1.9
10	5.6	13.6	16.0	12.2	13.5	12.2	1.8
20	9.6	12.0	9.6	14.2	22.4	13.6	2.4
30	7.2	10.4	26.4	13.8	21.3	15.8	3.5
40	9.6	26.4	35.2	14.0	26.0	22.2	4.6

TNF-a (2 hrs)	Experiment Number						
Perfusion Time (min)	S19	S22	S26	S33	S69	Average	SEM
1	0.7	6.7	1.8	0.0	0.0	1.8	1.3
3	1.5	4.7	8.7	3.6	4.8	4.6	1.2
5	4.4	13.8	7.2	13.3	23.5	12.4	3.3
10	6.4	30.4	21.6	13.3	19.0	18.1	4.0
20	15.2	52.0	28.8	26.0	34.3	31.3	6.0
30	22.4	47.2	32.0	33.8	29.1	32.9	4.1
40	12.2	56.8	41.6	39.4	33.0	36.6	7.2

Table B.28 Effect of forskolin on sickle erythrocyte adhesion induced by 6 hrs TNF-a stimulation. Average data are presented graphically in Figure 5.7, Panel A.

Unstimulated	Experiment Number											
Perfusion Time (min)	S21	S24	S25	S29	S30	S31	S50	S51	S61	S72	Average	SEM
1	4.4	2.5	3.2	1.4	3.8	0.8	0.9	5.8	2.3	1.1	2.6	0.5
3	4.8	6.7	1.4	4.5	5.3	4.0	3.6	6.7	7.6	4.6	4.9	0.6
5	6.4	28.4	3.2	5.6	5.1	5.3	6.4	13.0	12.8	12.8	9.9	2.4
10	16.0	27.2	0.0	1.6	5.9	22.7	2.5	18.9	14.6	16.0	12.5	3.0
20	23.2	36.0	0.8	8.8	6.4	11.8	7.0	16.0	7.0	21.3	13.8	3.3
30	22.5	59.2	0.8	11.4	10.4	17.6	1.3	13.3	8.7	27.4	17.3	5.4
40	14.5	52.8	1.6	2.4	6.4	15.2	3.0	14.6	13.0	30.7	15.4	5.0

Fsk (pre) + TNF-a (6 hrs)	Experiment Number											
Perfusion Time (min)	S21	S24	S25	S29	S30	S31	S50	S51	S61	S72	Average	SEM
1	1.5	5.1	7.4	0.7	0.8	8.6	2.3	3.4			3.7	1.1
3	9.3	13.1	14.9	10.0	7.7	6.7	0.8	13.3			9.5	1.6
5	18.6	41.9	12.7	17.6	21.1	6.2	5.1	16.7			17.5	4.0
10	25.6	56.0	16.0	21.6	31.2	17.6	7.3	19.6			24.4	5.2
20	63.2	92.8	34.4	31.2	43.6	27.4	17.5	37.1			43.4	8.5
30	107.2	95.2	33.9	45.6	91.2	39.2	18.0	41.9			59.0	11.8
40	157.7	120.8	32.8	60.8		48.8	25.9	58.4			72.2	18.4

Data are continued on the following page.

Table B.28, continued.

Fsk (pre + cont) + TNF-a (6 hrs)	Experiment Number											
Perfusion Time (min)	S21	S24	S25	S29	S30	S31	S50	S51	S61	S72	Average	SEM
1	3.2	4.7	0.0	0.6	0.8	0.8	2.9	3.5	3.5	0.6	2.1	0.5
3	15.2	16.0	4.2	2.8	4.5	6.4	4.0	10.4	6.9	13.9	8.4	1.6
5	22.1	21.8	4.8	7.2	19.2	5.3	7.1	6.2	5.3	17.8	11.7	2.4
10	31.3	34.4	16.0	16.0	20.0	5.6	12.8	10.4	12.4	27.8	18.7	3.0
20	32.7	63.2	17.6	17.6	30.4	2.4	14.1	26.7	12.5	35.8	25.3	5.3
30	57.6	72.8	9.6	43.4	46.4	12.0	12.2	35.0	24.3	34.9	34.8	6.6
40	109.6	109.6	17.6	36.0	43.2	7.3	13.0	40.0	25.5	34.9	43.7	11.6

TNF-a (6 hrs)	Experiment Number											
Perfusion Time (min)	S21	S24	S25	S29	S30	S31	S50	S51	S61	S72	Average	SEM
1	4.8	4.2	3.8	2.5	1.1	9.6	1.3	6.4	2.9	5.6	4.2	0.8
3	12.7	23.6	8.8	17.5	0.0	11.8	7.2	20.4	3.7	10.2	11.6	2.3
5	16.9	45.1	21.2	28.8	14.2	10.0	11.3	19.6	11.6	24.4	20.3	3.4
10	27.8	54.4	18.4	57.1	32.8	22.4	15.3	27.1	13.9	56.0	32.5	5.4
20	74.4	116.8	30.4	45.6	37.6	24.0	23.6	41.9	20.4	75.3	49.0	9.8
30	103.2	121.6	49.6	58.4	66.4	31.2	22.3	54.9	38.2	115.0	66.1	11.2
40	136.0	146.4	76.8	84.0	108.0	54.9	34.1	56.7	42.7	97.1	83.7	12.1

Table B.29 Effect of IBMX or Bt_2cAMP on sickle erythrocyte adhesion induced by 6 hrs TNF-a stimulation. Average data are presented graphically in Figure 5.7, Panels B and C.

Unstimulated	Experiment Number									
Perfusion Time (min)	S52	S54	S55	S57	S58	S59	S60	S61	Average	SEM
1	3.0	4.2	0.0	0.0	0.8	0.0	0.0	2.3	**1.3**	**0.6**
3	5.6	0.6	2.0	4.0	0.8	3.6	6.5	7.6	**3.9**	**0.9**
5	11.2	2.6	4.0	16.0	3.0	2.9	4.9	12.8	**7.2**	**1.9**
10	14.0	0.7	3.0	15.2	3.6	4.7	8.0	14.6	**8.0**	**2.1**
20	10.9	1.4	4.4	22.2	9.9	9.4	11.9	7.0	**9.6**	**2.2**
30	15.3	4.2	4.7	19.0	8.3	8.9	7.0	8.7	**9.5**	**1.8**
40	16.0	0.7	2.8	14.5	6.4	8.0	13.6	13.0	**9.4**	**2.0**

IBMX (pre + cont) + TNF-a (6 hrs)	Experiment Number									
Perfusion Time (min)	S52	S54	S55	S57	S58	S59	S60	S61	Average	SEM
1	5.3	2.7	0.7	4.0	3.3	5.8	2.8	3.5	**3.5**	**0.6**
3	12.0	4.1	3.8	6.7	2.4	1.2	9.4	3.2	**5.4**	**1.3**
5	19.0	5.7	8.0	8.4	5.9	5.3	5.8	6.0	**8.0**	**1.6**
10	27.3	13.8	21.6	12.4	7.5	5.9	12.0	15.4	**14.5**	**2.5**
20	45.3	16.6	28.4	13.9	3.6	6.4	18.7	21.6	**19.3**	**4.7**
30	53.5	21.6	23.7	13.3	9.5	12.2	21.3	27.1	**22.8**	**4.9**
40	48.0	16.0	25.7	21.8	18.3	11.1	17.4	23.7	**22.7**	**4.0**

Data are continued on the following page.

Table B.29, continued.

Bt_2cAMP (pre + cont) + TNF-a (6 hrs)	Experiment Number									
Perfusion Time (min)	S52	S54	S55	S57	S58	S59	S60	S61	Average	SEM
1	1.1	5.1	1.9	2.7	1.3	0.9	2.2	1.3	**2.1**	**0.5**
3	16.0	3.4	2.2	2.9	3.1	2.5	4.2	4.8	**4.9**	**1.6**
5	44.8	11.9	8.3	3.8	2.6	1.3	6.3	10.1	**11.1**	**5.0**
10	34.8	11.6	13.0	9.6	2.5	3.0	7.7	13.9	**12.0**	**3.6**
20	36.4	19.8	17.4	8.3	7.4	14.2	11.7	20.1	**16.9**	**3.3**
30	69.6	20.4	35.0	10.9	8.0	16.0	7.3	25.0	**24.0**	**7.3**
40	47.1	24.3	34.2	16.7	8.0	13.8	14.1	24.7	**22.9**	**4.5**

TNF-a (6 hrs)	Experiment Number									
Perfusion Time (min)	S52	S54	S55	S57	S58	S59	S60	S61	Average	SEM
1	12.3	4.2	3.4	5.3	1.5	0.0	3.6	2.9	**4.2**	**1.3**
3	38.7	2.2	8.0	8.0	6.0	2.5	12.7	3.7	**10.2**	**4.2**
5	27.2	5.6	9.6	12.0	7.4	5.0	10.0	11.6	**11.0**	**2.5**
10	60.6	17.2	16.0	10.2	12.5	18.6	33.3	13.9	**22.8**	**5.9**
20	81.0	24.3	19.6	20.9	13.7	16.8	39.0	20.4	**29.5**	**7.8**
30	111.1	46.1	32.0	26.9	14.5	28.0	33.9	38.2	**41.3**	**10.5**
40	125.2	42.4	29.0	30.7	17.4	31.2	53.6	42.7	**46.5**	**11.9**

Table B.30 Effect of forskolin on endothelial cAMP concentration. Data are given as pmol cAMP per well. Average data are presented graphically in Figures 5.8 and 5.9.

Endothelial Treatment	Experiment Number C1	C2	C3	Average	SEM
Unstimulated	1.08	2.76	2.85	2.23	0.57
Fsk (pre)	1.30	8.70	16.76	8.92	4.47
Fsk (pre) + TNF-a (0.5 hrs)	1.28	3.39	3.89	2.85	0.80
Fsk (pre) + TNF-a (1 hr)	1.22	3.30	4.01	2.84	0.84
Fsk (pre) + TNF-a (2 hrs)	1.25	2.33	3.11	2.23	0.54
Fsk (pre) + TNF-a (4 hrs)	1.40	2.53	2.85	2.26	0.44
Fsk (pre) + TNF-a (6 hrs)	1.46	2.10	4.11	2.55	0.80

Endothelial Treatment	Experiment Number C1	C2	C3	Average	SEM
Unstimulated	1.08	2.76	2.85	2.23	0.57
Fsk (pre)	1.30	8.70	16.76	8.92	4.47
Fsk (pre + cont) + TNF-a (0.5 hrs)	1.52	8.34	9.36	6.41	2.46
Fsk (pre + cont) + TNF-a (1 hr)	1.24	6.34	9.42	5.67	2.38
Fsk (pre + cont) + TNF-a (2 hrs)	1.03	3.71	6.52	3.75	1.59
Fsk (pre + cont) + TNF-a (4 hrs)	1.63	4.28	8.59	4.83	2.03
Fsk (pre + cont) + TNF-a (6 hrs)	1.82	5.17	11.20	6.06	2.74

Table B.31 Effect of forskolin alone on sickle erythrocyte adhesion. Average data are presented in Figure 5.10, Panel A.

Unstimulated	Experiment Number					
Perfusion Time (min)	S21	S22	S23	S24	Average	SEM
1	4.4	0.0	4.0	2.5	2.7	1.0
3	4.8	0.0	6.7	6.7	4.5	1.6
5	6.4	5.3	8.3	28.4	12.1	5.5
10	16.0	5.6	11.3	27.2	15.0	4.6
20	23.2	9.6	8.0	36.0	19.2	6.6
30	22.5	6.4	16.0	59.2	26.0	11.5
40	14.5	9.6	14.7	52.8	22.9	10.0

Fsk	Experiment Number					
Perfusion Time (min)	S21	S22	S23	S24	Average	SEM
1	0.0	0.0	6.9	0.0	1.7	1.7
3	1.5	5.3	7.4	9.8	6.0	1.8
5	0.6	7.1	11.3	13.8	8.2	2.9
10	3.2	1.6	18.4	20.9	11.0	5.0
20	4.0	11.2	19.8	23.2	14.6	4.3
30	8.8	8.8	21.1	40.8	19.9	7.6
40	4.8	4.8	19.2	22.9	12.9	4.7

TNF-^{a} (6 hrs)	Experiment Number					
Perfusion Time (min)	S21	S22	S23	S24	Average	SEM
1	4.8		5.9	4.2	5.0	0.5
3	12.7		21.3	23.6	19.2	3.3
5	16.9		69.1	45.1	43.7	15.1
10	27.8		129.6	54.4	70.6	30.5
20	74.4		220.8	116.8	137.3	43.5
30	103.2		251.2	121.6	158.7	46.6
40	136.0		333.6	146.4	205.3	64.2

Table B.32 Effect of IBMX and Bt_2cAMP alone on sickle erythrocyte adhesion. Average data are presented in Figure 5.10, Panel B.

Unstimulated	Experiment Number				
Perfusion Time (min)	S58	S59	S63	Average	SEM
1	0.8	0.0	0.0	0.3	0.3
3	0.8	3.6	8.0	4.2	2.1
5	3.0	2.9	6.2	4.0	1.1
10	3.6	4.7	21.6	10.0	5.8
20	9.9	9.4	23.0	14.1	4.4
30	8.3	8.9	24.5	13.9	5.3
40	6.4	8.0	26.0	13.5	6.3

IBMX	Experiment Number				
Perfusion Time (min)	S58	S59	S63	Average	SEM
1	2.4	3.7	0.0	2.0	1.1
3	3.6	10.3	8.0	7.3	2.0
5	6.7	14.5	12.0	11.1	2.3
10	5.8	13.9	16.0	11.9	3.1
20	6.5	16.0	28.0	16.8	6.2
30	9.7	6.4	28.3	14.8	6.8
40	9.7	8.7	32.0	16.8	7.6

Bt_2cAMP	Experiment Number				
Perfusion Time (min)	S58	S59	S63	Average	SEM
1	1.8	0.0	2.0	1.3	0.6
3	1.1	0.8	12.8	4.9	4.0
5	5.9	1.5	25.1	10.8	7.3
10	2.3	1.6	33.1	12.3	10.4
20	5.3	2.5	40.6	16.1	12.3
30	5.1	2.3	74.2	27.2	23.5
40	5.1	4.0	43.1	17.4	12.8

TNF-a (6 hrs)	Experiment Number				
Perfusion Time (min)	S58	S59	S63	Average	SEM
1	1.5	0.0	1.5	1.0	0.5
3	6.0	2.5	30.7	13.0	8.9
5	7.4	5.0	27.4	13.3	7.1
10	12.5	18.6	69.0	33.4	17.9
20	13.7	16.8	104.5	45.0	29.8
30	14.5	28.0	140.4	61.0	39.9
40	17.4	31.2	160.0	69.5	45.4

Table B.33 Effect of treatment of sickle erythrocytes with forskolin on adhesion to unstimulated MECs. Data are presented graphically in Figure 5.11, Panel A.

Unstimulated SSRBCs Unstimulated MECs	Experiment Number					
Perfusion Time (min)	S61	S64	S67	S72	Average	SEM
1	2.3	1.7	0.8	1.1	1.5	0.3
3	7.6	4.4	7.0	4.6	5.9	0.8
5	12.8	5.3	12.6	12.8	10.9	1.8
10	14.6	10.9	17.7	16.0	14.8	1.4
20	7.0	14.5	14.5	21.3	14.3	2.9
30	8.7	10.7	16.6	27.4	15.9	4.2
40	13.0	10.7	14.8	30.7	17.3	4.5

Fsk on SSRBCs Unstimulated MECs	Experiment Number					
Perfusion Time (min)	S61	S64	S67	S72	Average	SEM
1	0.7	1.5	4.6	1.9	2.2	0.8
3	2.0	4.4	5.1	6.6	4.5	1.0
5	8.7	13.3	9.0	16.8	12.0	1.9
10	11.3	22.0	16.0	24.0	18.3	2.9
20	9.7	31.3	26.4	39.6	26.8	6.3
30	12.5	36.7	15.1	42.0	26.6	7.5
40	14.0	36.3	25.6	27.0	25.7	4.6

Table B.34 Effect of treatment of sickle erythrocytes with IBMX and Bt_2cAMP on adhesion to unstimulated MECs. Data are presented graphically in Figure 5.11, Panels B and C.

Unstimulated SSRBCs Unstimulated MECs	Experiment Number				
Perfusion Time (min)	S61	S67	S72	Average	SEM
1	2.3	0.8	1.1	1.4	0.4
3	7.6	7.0	4.6	6.4	0.9
5	12.8	12.6	12.8	12.7	0.1
10	14.6	17.7	16.0	16.1	0.9
20	7.0	14.5	21.3	14.3	4.2
30	8.7	16.6	27.4	17.6	5.4
40	13.0	14.8	30.7	19.5	5.6

IBMX on SSRBCs Unstimulated MECs	Experiment Number				
Perfusion Time (min)	S61	S67	S72	Average	SEM
1	3.2	2.3	2.0	2.5	0.4
3	4.8	5.1	6.3	5.4	0.4
5	6.0	19.2	9.1	11.4	4.0
10	9.3	29.1	18.4	18.9	5.7
20	12.5	35.2	48.8	32.2	10.6
30	13.4	38.9	22.7	25.0	7.4
40	11.3	42.2	42.4	32.0	10.3

Bt_2cAMP on SSRBCs Unstimulated MECs	Experiment Number				
Perfusion Time (min)	S61	S67	S72	Average	SEM
1	3.0	1.4	0.0	1.5	0.9
3	2.4	8.8	10.4	7.2	2.5
5	4.9	14.4	14.3	11.2	3.1
10	5.1	18.0	20.8	14.6	4.8
20	7.3	34.9	48.0	30.1	12.0
30	13.2	24.7	29.7	22.6	4.9
40	11.1	14.2	43.3	22.9	10.2

Table B.35 Effect of treatment of sickle erythrocytes with forskolin on adhesion to MECs stimulated with TNF-a for 6 hrs. Data are presented graphically in Figure 5.12, Panel A.

Unstimulated SSRBCs Unstimulated MECs	Experiment Number					
Perfusion Time (min)	S61	S64	S67	S72	Average	SEM
1	2.3	1.7	0.8	1.1	1.5	0.3
3	7.6	4.4	7.0	4.6	5.9	0.8
5	12.8	5.3	12.6	12.8	10.9	1.8
10	14.6	10.9	17.7	16.0	14.8	1.4
20	7.0	14.5	14.5	21.3	14.3	2.9
30	8.7	10.7	16.6	27.4	15.9	4.2
40	13.0	10.7	14.8	30.7	17.3	4.5

Fsk on SSRBCs TNF-a (6 hrs) on MECs	Experiment Number					
Perfusion Time (min)	S61	S64	S67	S72	Average	SEM
1	2.8	0.0	1.6	3.2	1.9	0.7
3	11.5	5.1	5.6	7.7	7.5	1.5
5	17.4	5.1	28.8	32.0	20.8	6.1
10	36.6	10.9	26.7	80.9	38.8	15.0
20	43.1	14.7	43.2	88.0	47.2	15.2
30	55.3	22.0	43.1	84.6	51.2	13.1
40	64.7	18.2	41.0	111.0	58.7	19.8

Unstimulated SSRBCs TNF-a (6 hrs) on MECs	Experiment Number					
Perfusion Time (min)	S61	S64	S67	S72	Average	SEM
1	2.9	0.0	0.8	5.6	2.3	1.2
3	3.7	2.8	6.3	10.2	5.7	1.7
5	11.6	23.4	13.9	24.4	18.3	3.2
10	13.9	14.7	13.6	56.0	24.5	10.5
20	20.4	29.2	18.5	75.3	35.8	13.4
30	38.2	24.3	22.4	115.0	50.0	22.0
40	42.7	25.6	37.8	97.1	50.8	15.8

Table B.36 Effect of treatment of sickle erythrocytes with Bt_2cAMP on adhesion to MECs stimulated with TNF-a for 6 hrs. Data are presented graphically in Figure 5.12, Panel B.

Unstimulated SSRBCs Unstimulated MECs	Experiment Number				
Perfusion Time (min)	S61	S67	S72	Average	SEM
1	2.3	0.8	1.1	1.4	0.4
3	7.6	7.0	4.6	6.4	0.9
5	12.8	12.6	12.8	12.7	0.1
10	14.6	17.7	16.0	16.1	0.9
20	7.0	14.5	21.3	14.3	4.2
30	8.7	16.6	27.4	17.6	5.4
40	13.0	14.8	30.7	19.5	5.6

Bt2cAMP on SSRBCs TNF-a (6 hrs) on MECs	Experiment Number				
Perfusion Time (min)	S61	S67	S72	Average	SEM
1	3.2	4.4	1.9	3.1	0.7
3	3.6	12.8	20.0	12.1	4.8
5	6.0	20.6	38.7	21.8	9.5
10	5.0	44.8	56.0	35.3	15.5
20	4.9	22.2	55.3	27.5	14.8
30	12.5	24.6	83.0	40.1	21.8
40	13.8	48.0	73.0	44.9	17.2

Unstimulated SSRBCs TNF-a (6 hrs)	Experiment Number				
Perfusion Time (min)	S61	S67	S72	Average	SEM
1	2.9	0.8	5.6	3.1	1.4
3	3.7	6.3	10.2	6.7	1.9
5	11.6	13.9	24.4	16.7	3.9
10	13.9	13.6	56.0	27.8	14.1
20	20.4	18.5	75.3	38.0	18.6
30	38.2	22.4	115.0	58.5	28.6
40	42.7	37.8	97.1	59.2	19.0

Table B.37 Effect of blood age on sickle erythrocyte adhesion to unstimulated and TNF-a stimulated MECs. Data are presented graphically in Figures 5.13 through 5.15.

	Adherent Erythrocytes / mm^2	
Experiment Number	Unstimulated	TNF-a (6 hrs)
S19	2.4	40.8
S21	14.5	136.0
S24	52.8	146.4
S25	1.6	76.8
S28	6.4	17.2
S29	2.4	84.0
S30	6.4	108.0
S31	15.2	54.9
S34	3.3	80.8
S35	2.4	17.2
S40	13.7	35.8
S42	7.6	49.8
S43	23.6	68.4
S45	1.5	22.1
S46	15.2	137.1
S47	18.3	46.3
S50	3.0	34.1
S51	14.6	56.7
S52	16.0	125.2
S53	15.3	63.3
S54	0.7	42.4
S55	2.8	29.0
S57	14.5	30.7
S58	6.4	17.4
S59	8.0	31.2
S60	13.6	53.6
S61	13.0	42.7
S62	16.0	29.1
S63	26.0	160.0
S64	10.7	25.6
S65	16.6	54.7
S66	14.2	24.5
S67	14.8	37.8
S68	6.2	56.9
S70	8.4	24.6
S71	17.2	46.6
S72	30.7	97.1
S73	54.4	31.2
S74	27.4	42.3
S75	12.4	52.0
S76	18.8	81.0
S77	11.6	29.9
S78	24.8	52.4

Table B.38 Inhibition of TNF-a induced adhesion molecule expression by SNP. Average data are presented graphically in Figure 6.1, Panel A.

VCAM-1	Experiment Number							
Endothelial Treatment	**E12**	**E14**	**E26**	**E28**	**E29**	**E30**	**Average**	**SEM**
Unstimulated	0.047	0.000	0.000	0.000	0.000	0.000	0.008	0.008
TNF-a (6 hrs)	0.286	0.165	0.260	0.233	0.215	0.302	0.243	0.021
SNP (pre + cont) + TNF-a (6 hrs)	0.153	0.165	0.000	0.115	0.048	0.087	0.095	0.026

E-selectin	Experiment Number							
Endothelial Treatment	**E12**	**E14**	**E26**	**E28**	**E29**	**E30**	**Average**	**SEM**
Unstimulated	0.045	0.000	0.000	0.000	0.002	0.331	0.063	0.054
TNF-a (6 hrs)	0.628	0.594	0.667	0.640	0.664	0.793	0.664	0.028
SNP (pre + cont) + TNF-a (6 hrs)	0.427	0.593	0.000	0.606	0.422	0.706	0.459	0.102

ICAM-1	Experiment Number							
Endothelial Treatment	**E12**	**E14**	**E26**	**E28**	**E29**	**E30**	**Average**	**SEM**
Unstimulated	0.291	0.183	0.207	0.162	0.145	0.204	0.199	0.021
TNF-a (6 hrs)	1.051	0.838	0.900	0.781	0.817	0.877	0.877	0.039
SNP (pre + cont) + TNF-a (6 hrs)	1.120	0.673	0.174	0.699	0.621	0.714	0.667	0.123

Table B.39 Inhibition of TNF-a induced adhesion molecule expression by DETA-NO. Average data are presented graphically in Figure 6.1, Panel B.

VCAM-1	Experiment Number					
Endothelial Treatment	**E14**	**E26**	**E29**	**E30**	**Average**	**SEM**
Unstimulated	0.000	0.000	0.000	0.000	0.000	0.000
TNF-a (6 hrs)	0.139	0.260	0.193	0.281	0.218	0.032
DETA-NO (pre + cont) + TNF-a (6 hrs)	0.037	0.015	0.054	0.097	0.051	0.017

E-selectin	Experiment Number					
Endothelial Treatment	**E14**	**E26**	**E29**	**E30**	**Average**	**SEM**
Unstimulated	0.000	0.000	0.000	0.000	0.000	0.000
TNF-a (6 hrs)	0.175	0.667	0.569	0.712	0.531	0.122
DETA-NO (pre + cont) + TNF-a (6 hrs)	0.081	0.009	0.436	0.533	0.265	0.129

ICAM-1	Experiment Number					
Endothelial Treatment	**E14**	**E26**	**E29**	**E30**	**Average**	**SEM**
Unstimulated	0.174	0.207	0.150	0.091	0.156	0.024
TNF-a (6 hrs)	0.000	0.900	0.766	0.774	0.610	0.206
DETA-NO (pre + cont) + TNF-a (6 hrs)	0.061	0.525	0.703	0.743	0.508	0.156

Table B.40 Adhesion molecule expression on MECs treated with SNP alone. Average data are presented in Table 6.1.

VCAM-1	Experiment Number				
Endothelial Treatment	**E14**	**E28**	**E17**	**Average**	**SEM**
Unstimulated	0.000	0.000	0.034	0.011	0.011
SNP	0.000	0.000	0.022	0.007	0.007
TNF-α	0.165	0.233	0.044	0.147	0.055

E-selectin	Experiment Number				
Endothelial Treatment	**E14**	**E28**	**E17**	**Average**	**SEM**
Unstimulated	0.000	0.000	0.063	0.021	0.021
SNP	0.000	0.000	0.132	0.044	0.044
TNF-α	0.594	0.640	0.206	0.480	0.138

ICAM-1	Experiment Number				
Endothelial Treatment	**E14**	**E28**	**E17**	**Average**	**SEM**
Unstimulated	0.183	0.162	0.199	0.182	0.011
SNP	0.180	0.064	0.133	0.126	0.034
TNF-α	0.838	0.781	0.416	0.678	0.132

Table B.41 Adhesion molecule expression on MECs treated with DETA-NO alone. Average data are presented in Table 6.1.

VCAM-1	Experiment Number				
Endothelial Treatment	**E14**	**E28**	**E18**	**Average**	**SEM**
Unstimulated	0.000	0.000	0.024	0.008	0.008
DETA-NO	0.000	0.000	0.048	0.016	0.016
TNF-α	0.193	0.281	0.088	0.187	0.056

E-selectin	Experiment Number				
Endothelial Treatment	**E14**	**E28**	**E18**	**Average**	**SEM**
Unstimulated	0.000	0.000	0.099	0.033	0.033
DETA-NO	0.000	0.000	0.104	0.035	0.035
TNF-α	0.569	0.712	0.462	0.581	0.072

ICAM-1	Experiment Number				
Endothelial Treatment	**E14**	**E28**	**E18**	**Average**	**SEM**
Unstimulated	0.150	0.091	0.286	0.176	0.058
DETA-NO	0.275	0.244	0.468	0.329	0.070
TNF-α	0.766	0.774	0.549	0.696	0.074

Table B.42 Inhibition of TNF-a induced sickle erythrocyte adhesion by SNP. Average data are presented graphically in Figure 6.2, Panel A.

Unstimulated	Experiment Number											
Perfusion Time (min)	**S34**	**S35**	**S49**	**S52**	**S57**	**S66**	**S73**	**S74**	**S75**	**S76**	**Average**	**SEM**
1	0.0	0.7	10.1	3.0	0.0	0.8	0.0	3.7	2.1	0.0	**2.1**	**1.0**
3	1.8	0.0	5.1	5.6	4.0	4.4	9.8	16.0	8.0	8.0	**6.3**	**1.4**
5	1.6	2.5	5.5	11.2	16.0	9.3	14.2	12.0	9.1	4.4	**8.6**	**1.6**
10	4.2	2.4	8.0	14.0	15.2	11.1	29.1	35.2	12.4	14.6	**14.6**	**3.3**
20	6.7	4.8	5.3	10.9	22.2	11.1	39.0	24.0	9.8	16.0	**15.0**	**3.4**
30	6.4	2.4	2.1	15.3	19.0	12.5	35.0	43.2	9.6	12.8	**15.8**	**4.3**
40	3.3	2.4	8.0	16.0	14.5	14.2	54.4	27.4	12.4	18.8	**17.1**	**4.7**

TNF-a (6 hrs)	Experiment Number											
Perfusion Time (min)	**S34**	**S35**	**S49**	**S52**	**S57**	**S66**	**S73**	**S74**	**S75**	**S76**	**Average**	**SEM**
1	0.0	3.2	3.4	12.3	5.3	2.2	1.5	2.3	0.8	8.8	**4.0**	**1.2**
3	9.5	7.2	18.2	38.7	8.0	2.2	2.3	16.8	5.6	17.6	**12.6**	**3.5**
5	17.7	9.1	20.8	27.2	12.0	7.6	30.7	37.7	9.0	36.7	**20.9**	**3.7**
10	31.2	9.3	32.7	60.6	10.2	17.4	29.9	74.7	25.3	34.2	**32.5**	**6.6**
20	72.8	20.0	37.8	81.0	20.9	35.2	32.0	59.1	69.3	48.8	**47.7**	**6.9**
30	84.2	15.2	41.7	111.1	26.9	28.2	21.7	31.3	82.0	75.2	**51.8**	**10.5**
40	80.8	17.2	41.7	125.2	30.7	24.5	31.2	42.3	52.0	81.0	**52.7**	**10.6**

SNP (pre + cont) + TNF-a (6 hrs)	Experiment Number											
Perfusion Time (min)	**S34**	**S35**	**S49**	**S52**	**S57**	**S66**	**S73**	**S74**	**S75**	**S76**	**Average**	**SEM**
1	3.6	0.8	6.2	2.1	2.3	0.8	0.0	1.9	4.2	1.8	**2.4**	**0.6**
3	3.5	2.2	14.3	13.0	5.0	2.4	8.7	7.2	12.6	9.1	**7.8**	**1.4**
5	5.6	7.2	12.0	24.8	9.0	6.4	8.0	28.6	29.0	8.6	**13.9**	**3.0**
10	5.6	6.4	21.3	27.1	11.8	20.4	18.7	39.5	16.0	21.1	**18.8**	**3.2**
20	2.9	13.8	22.3	32.0	15.3	18.3	29.0	34.0	31.0	27.1	**22.6**	**3.1**
30	9.3	28.6	37.3	61.4	10.9	23.4	26.1	35.0	26.3	21.6	**28.0**	**4.7**
40	5.3	25.6	41.8	56.0	10.4	20.9	22.9	29.9	28.8	32.7	**27.4**	**4.6**

Table B.43 Inhibition of TNF-a induced sickle erythrocyte adhesion by DETA-NO. Average data are presented graphically in Figure 6.2, Panel B.

Unstimulated	Experiment Number												
Perfusion Time (min)	**S49**	**S52**	**S57**	**S62**	**S66**	**S70**	**S71**	**S73**	**S74**	**S75**	**S76**	**Average**	**SEM**
1	10.1	3.0	0.0	2.5	0.8	3.4	1.0	0.0	3.7	2.1	0.0	**2.4**	**0.9**
3	5.1	5.6	4.0	3.4	4.4	6.9	3.6	9.8	16.0	8.0	8.0	**6.8**	**1.1**
5	5.5	11.2	16.0	4.8	9.3	9.3	6.4	14.2	12.0	9.1	4.4	**9.3**	**1.2**
10	8.0	14.0	15.2	8.0	11.1	3.8	4.2	29.1	35.2	12.4	14.6	**14.1**	**3.0**
20	5.3	10.9	22.2	9.6	11.1	5.8	13.2	39.0	24.0	9.8	16.0	**15.2**	**3.0**
30	2.1	15.3	19.0	5.1	12.5	3.8	21.6	35.0	43.2	9.6	12.8	**16.4**	**3.9**
40	8.0	16.0	14.5	16.0	14.2	8.4	17.2	54.4	27.4	12.4	18.8	**18.9**	**3.9**

TNF-a (6 hrs)	Experiment Number												
Perfusion Time (min)	**S49**	**S52**	**S57**	**S62**	**S66**	**S70**	**S71**	**S73**	**S74**	**S75**	**S76**	**Average**	**SEM**
1	3.4	12.3	5.3	0.7	2.2	4.2	1.6	1.5	2.3	0.8	8.8	**3.9**	**1.1**
3	18.2	38.7	8.0	4.7	2.2	6.1	7.0	2.3	16.8	5.6	17.6	**11.5**	**3.3**
5	20.8	27.2	12.0	5.9	7.6	4.8	5.6	30.7	37.7	9.0	36.7	**18.0**	**3.9**
10	32.7	60.6	10.2	14.2	17.4	13.2	11.6	29.9	74.7	25.3	34.2	**29.4**	**6.3**
20	37.8	81.0	20.9	9.7	35.2	21.6	16.0	32.0	59.1	69.3	48.8	**39.2**	**6.9**
30	41.7	111.1	26.9	23.3	28.2	19.0	28.5	21.7	31.3	82.0	75.2	**44.5**	**9.2**
40	41.7	125.2	30.7	29.1	24.5	24.6	46.6	31.2	42.3	52.0	81.0	**48.1**	**9.1**

DETA-NO (pre + cont) + TNF-a (6 hrs)	Experiment Number												
Perfusion Time (min)	**S49**	**S52**	**S57**	**S62**	**S66**	**S70**	**S71**	**S73**	**S74**	**S75**	**S76**	**Average**	**SEM**
1	10.0	1.7	2.1	0.6	1.0	0.0	3.2	1.0	2.7	0.0	1.1	**2.1**	**0.8**
3	6.7	8.0	4.5	0.7	10.2	11.6	4.8	11.1	6.1	3.2	6.4	**6.7**	**1.0**
5	10.4	21.6	6.2	5.7	11.6	8.0	10.4	23.3	12.8	8.8	13.1	**12.0**	**1.7**
10	14.0	47.1	6.8	6.0	16.0	3.8	11.9	18.3	28.8	18.7	12.4	**16.7**	**3.7**
20	20.7	34.9	13.2	7.0	28.4	13.9	23.3	31.0	57.6	23.7	21.7	**25.0**	**4.1**
30	24.4	42.4	9.1	8.3	27.6	17.0	10.9	48.0	44.0	20.2	22.7	**25.0**	**4.3**
40	25.5	55.0	9.0	2.8	40.0	15.0	23.3	15.2	46.4	26.7	15.1	**24.9**	**4.9**

Table B.44 Effect of SNP alone on sickle erythrocyte adhesion. Average data are presented graphically in Figure 6.3, Panel A.

Unstimulated	Experiment Number									
Perfusion Time (min)	S62	S70	S71	S73	S74	S75	S77	S78	Average	SEM
1	2.5	3.4	1.0	0.0	3.7	2.1	3.2	0.0	**2.0**	**0.5**
3	3.4	6.9	3.6	9.8	16.0	8.0	5.3	10.7	**8.0**	**1.5**
5	4.8	9.3	6.4	14.2	12.0	9.1	2.4	9.8	**8.5**	**1.4**
10	8.0	3.8	4.2	29.1	35.2	12.4	10.1	14.4	**14.6**	**4.1**
20	9.6	5.8	13.2	39.0	24.0	9.8	9.6	17.5	**16.1**	**3.8**
30	5.1	3.8	21.6	35.0	43.2	9.6	8.3	18.3	**18.1**	**5.1**
40	16.0	8.4	17.2	54.4	27.4	12.4	11.6	24.8	**21.5**	**5.2**

TNF-a (6 hrs)	Experiment Number									
Perfusion Time (min)	S62	S70	S71	S73	S74	S75	S77	S78	Average	SEM
1	0.7	4.2	1.6	1.5	2.3	0.8	0.9	3.5	**1.9**	**0.5**
3	4.7	6.1	7.0	2.3	16.8	5.6	3.8	13.9	**7.5**	**1.8**
5	5.9	4.8	5.6	30.7	37.7	9.0	6.0	22.9	**15.3**	**4.7**
10	14.2	13.2	11.6	29.9	74.7	25.3	16.0	25.6	**26.3**	**7.3**
20	9.7	21.6	16.0	32.0	59.1	69.3	15.3	36.6	**32.5**	**7.7**
30	23.3	19.0	28.5	21.7	31.3	82.0	27.1	41.6	**34.3**	**7.2**
40	29.1	24.6	46.6	31.2	42.3	52.0	29.9	52.4	**38.5**	**3.9**

SNP	Experiment Number									
Perfusion Time (min)	S62	S70	S71	S73	S74	S75	S77	S78	Average	SEM
1	1.8	1.8	4.4	0.0	0.8	0.6	3.6	3.3	**2.0**	**0.6**
3	3.2	4.6	4.4	4.0	5.3	8.4	10.0	7.1	**5.9**	**0.8**
5	4.9	1.5	11.1	14.5	12.2	5.3	11.8	20.4	**10.2**	**2.1**
10	9.6	9.9	8.3	17.4	11.3	26.9	11.6	33.7	**16.1**	**3.3**
20	10.2	12.5	19.5	11.1	12.5	24.4	10.7	32.8	**16.7**	**2.9**
30	19.8	15.3	20.4	19.0	7.7	16.5	13.0	49.1	**20.1**	**4.4**
40	7.0	10.0	17.5	21.9	6.4	16.8	11.6	23.3	**14.3**	**2.3**

Table B.45 Effect of DETA-NO alone on sickle erythrocyte adhesion. Average data are presented graphically in Figure 6.3, Panel B.

Unstimulated	Experiment Number								
Perfusion Time (min)	S62	S63	S70	S71	S75	S77	S78	Average	SEM
1	2.5	0.0	3.4	1.0	2.1	3.2	0.0	**1.7**	**0.5**
3	3.4	8.0	6.9	3.6	8.0	5.3	10.7	**6.5**	**1.0**
5	4.8	6.2	9.3	6.4	9.1	2.4	9.8	**6.9**	**1.0**
10	8.0	21.6	3.8	4.2	12.4	10.1	14.4	**10.6**	**2.4**
20	9.6	23.0	5.8	13.2	9.8	9.6	17.5	**12.6**	**2.2**
30	5.1	24.5	3.8	21.6	9.6	8.3	18.3	**13.0**	**3.1**
40	16.0	26.0	8.4	17.2	12.4	11.6	24.8	**16.6**	**2.5**

TNF-a (6 hrs)	Experiment Number								
Perfusion Time (min)	S62	S63	S70	S71	S75	S77	S78	Average	SEM
1	0.7	1.5	4.2	1.6	0.8	0.9	3.5	**1.9**	**0.5**
3	4.7	30.7	6.1	7.0	5.6	3.8	13.9	**10.2**	**3.6**
5	5.9	27.4	4.8	5.6	9.0	6.0	22.9	**11.6**	**3.6**
10	14.2	69.0	13.2	11.6	25.3	16.0	25.6	**25.0**	**7.6**
20	9.7	104.5	21.6	16.0	69.3	15.3	36.6	**39.0**	**13.3**
30	23.3	140.4	19.0	28.5	82.0	27.1	41.6	**51.7**	**16.8**
40	29.1	160.0	24.6	46.6	52.0	29.9	52.4	**56.4**	**17.8**

DETA-NO	Experiment Number								
Perfusion Time (min)	S62	S63	S70	S71	S75	S77	S78	Average	SEM
1	0.0	4.4	4.4	0.0	1.5	0.0	3.6	**2.0**	**0.8**
3	2.6	11.0	6.2	2.1	6.4	5.3	9.1	**6.1**	**1.2**
5	3.0	23.1	13.2	1.4	14.3	4.7	13.3	**10.4**	**2.9**
10	13.8	19.4	8.0	11.3	14.2	10.4	19.8	**13.8**	**1.7**
20	13.8	39.5	16.0	10.0	24.7	6.6	28.4	**19.9**	**4.4**
30	16.0	42.4	12.6	11.6	15.2	21.3	38.9	**22.6**	**4.8**
40	7.7	40.7	17.8	12.9	24.0	8.0	43.1	**22.0**	**5.6**

Table B.46 Adhesion of sickle erythrocytes treated with SNP to unstimulated MECs. Average data are presented graphically in Figure 6.4, Panel A.

Unstimulated SSRBCs Unstimulated MECs	Experiment Number								
Perfusion Time (min)	**S52**	**S64**	**S65**	**S66**	**S73**	**S74**	**S75**	**Average**	**SEM**
1	3.0	1.7	0.0	0.8	0.0	3.7	2.1	**1.6**	**0.5**
3	5.6	4.4	3.2	4.4	9.8	16.0	8.0	**7.4**	**1.7**
5	11.2	5.3	2.9	9.3	14.2	12.0	9.1	**9.2**	**1.5**
10	14.0	10.9	8.0	11.1	29.1	35.2	12.4	**17.2**	**4.0**
20	10.9	14.5	4.1	11.1	39.0	24.0	9.8	**16.2**	**4.4**
30	15.3	10.7	11.6	12.5	35.0	43.2	9.6	**19.7**	**5.1**
40	16.0	10.7	16.6	14.2	54.4	27.4	12.4	**21.7**	**5.8**

Unstimulated SSRBCs TNF-a (6 hrs) on MECs	Experiment Number								
Perfusion Time (min)	**S52**	**S64**	**S65**	**S66**	**S73**	**S74**	**S75**	**Average**	**SEM**
1	12.3	0.0	1.5	2.2	1.5	2.3	0.8	**2.9**	**1.6**
3	38.7	2.8	9.3	2.2	2.3	16.8	5.6	**11.1**	**5.0**
5	27.2	23.4	8.5	7.6	30.7	37.7	9.0	**20.6**	**4.6**
10	60.6	14.7	20.4	17.4	29.9	74.7	25.3	**34.7**	**8.8**
20	81.0	29.2	32.0	35.2	32.0	59.1	69.3	**48.3**	**8.0**
30	111.1	24.3	51.0	28.2	21.7	31.3	82.0	**49.9**	**12.9**
40	125.2	25.6	54.7	24.5	31.2	42.3	52.0	**50.8**	**13.2**

SNP on SSRBCs Unstimulated MECs	Experiment Number								
Perfusion Time (min)	**S52**	**S64**	**S65**	**S66**	**S73**	**S74**	**S75**	**Average**	**SEM**
1	2.8	4.6	0.0	2.5	2.4	1.1	2.1	**2.2**	**0.5**
3	6.6	5.8	7.6	10.1	4.9	16.0	6.2	**8.2**	**1.5**
5	14.1	8.7	5.3	10.7	8.8	24.5	9.6	**11.7**	**2.4**
10	16.0	15.3	15.2	12.2	16.0	13.9	17.1	**15.1**	**0.6**
20	16.0	18.0	17.1	7.3	37.0	22.6	17.1	**19.3**	**3.4**
30	22.1	20.0	23.5	12.8	37.0	40.7	28.0	**26.3**	**3.7**
40	28.8	27.3	25.0	8.7	35.2	37.6	22.9	**26.5**	**3.6**

Table B.47 Adhesion of sickle erythrocytes treated with DETA-NO to unstimulated MECs. Average data are presented graphically in Figure 6.4, Panel B.

Unstimulated SSRBCs Unstimulated MECs	Experiment Number						
Perfusion Time (min)	S65	S66	S73	S74	S75	Average	SEM
1	0.0	0.8	0.0	3.7	2.1	**1.3**	**0.7**
3	3.2	4.4	9.8	16.0	8.0	**8.3**	**2.3**
5	2.9	9.3	14.2	12.0	9.1	**9.5**	**1.9**
10	8.0	11.1	29.1	35.2	12.4	**19.2**	**5.4**
20	4.1	11.1	39.0	24.0	9.8	**17.6**	**6.3**
30	11.6	12.5	35.0	43.2	9.6	**22.4**	**7.0**
40	16.6	14.2	54.4	27.4	12.4	**25.0**	**7.8**

Unstimulated SSRBCs TNF-a (6 hrs) on MECs	Experiment Number						
Perfusion Time (min)	S65	S66	S73	S74	S75	Average	SEM
1	1.5	2.2	1.5	2.3	0.8	**1.6**	**0.3**
3	9.3	2.2	2.3	16.8	5.6	**7.2**	**2.7**
5	8.5	7.6	30.7	37.7	9.0	**18.7**	**6.4**
10	20.4	17.4	29.9	74.7	25.3	**33.5**	**10.5**
20	32.0	35.2	32.0	59.1	69.3	**45.5**	**7.8**
30	51.0	28.2	21.7	31.3	82.0	**42.8**	**10.9**
40	54.7	24.5	31.2	42.3	52.0	**41.0**	**5.8**

DETA-NO on SSRBCs Unstimulated MECs	Experiment Number						
Perfusion Time (min)	S65	S66	S73	S74	S75	Average	SEM
1	2.8	0.0	0.6	1.1	4.8	**1.9**	**0.9**
3	1.6	5.3	8.3	19.6	5.1	**8.0**	**3.1**
5	3.8	10.7	9.1	38.9	7.6	**14.0**	**6.3**
10	8.4	10.4	20.0	27.6	7.1	**14.7**	**3.9**
20	12.0	20.8	10.5	26.9	20.0	**18.0**	**3.0**
30	11.1	10.9	22.9	29.5	25.1	**19.9**	**3.8**
40	18.9	11.6	16.9	52.0	27.0	**25.3**	**7.1**

Table B.48 Adhesion of sickle erythrocytes treated with SNP to TNF-α stimulated MECs. Average data are presented graphically in Figure 6.5, Panel A.

Unstimulated SSRBCs Unstimulated MECs	Experiment Number									
Perfusion Time (min)	**S49**	**S52**	**S73**	**S74**	**S75**	**S76**	**S77**	**S78**	**Average**	**SEM**
1	10.1	3.0	0.0	3.7	2.1	0.0	3.2	0.0	**2.8**	**1.2**
3	5.1	5.6	9.8	16.0	8.0	8.0	5.3	10.7	**8.6**	**1.3**
5	5.5	11.2	14.2	12.0	9.1	4.4	2.4	9.8	**8.6**	**1.4**
10	8.0	14.0	29.1	35.2	12.4	14.6	10.1	14.4	**17.2**	**3.4**
20	5.3	10.9	39.0	24.0	9.8	16.0	9.6	17.5	**16.5**	**3.8**
30	2.1	15.3	35.0	43.2	9.6	12.8	8.3	18.3	**18.1**	**5.0**
40	8.0	16.0	54.4	27.4	12.4	18.8	11.6	24.8	**21.7**	**5.2**

Unstimulated SSRBCs TNF-α (6 hrs) on MECs	Experiment Number									
Perfusion Time (min)	**S49**	**S52**	**S73**	**S74**	**S75**	**S76**	**S77**	**S78**	**Average**	**SEM**
1	3.4	12.3	1.5	2.3	0.8	8.8	0.9	3.5	**4.2**	**1.5**
3	18.2	38.7	2.3	16.8	5.6	17.6	3.8	13.9	**14.6**	**4.1**
5	20.8	27.2	30.7	37.7	9.0	36.7	6.0	22.9	**23.9**	**4.2**
10	32.7	60.6	29.9	74.7	25.3	34.2	16.0	25.6	**37.4**	**7.0**
20	37.8	81.0	32.0	59.1	69.3	48.8	15.3	36.6	**47.5**	**7.6**
30	41.7	111.1	21.7	31.3	82.0	75.2	27.1	41.6	**54.0**	**11.2**
40	41.7	125.2	31.2	42.3	52.0	81.0	29.9	52.4	**57.0**	**11.3**

SNP on SSRBCs TNF-α (6 hrs) on MECs	Experiment Number									
Perfusion Time (min)	**S49**	**S52**	**S73**	**S74**	**S75**	**S76**	**S77**	**S78**	**Average**	**SEM**
1	3.2	7.2	5.8	2.4	2.3	1.6	4.3	1.5	**3.5**	**0.7**
3	9.5	12.0	8.3	8.9	11.2	7.2	9.6	2.9	**8.7**	**1.0**
5	10.2	23.0	53.3	21.3	15.1	11.6	3.8	12.8	**18.9**	**5.4**
10	21.5	30.6	22.0	48.0	26.3	27.5	10.4	31.3	**27.2**	**3.8**
20	34.2	28.5	39.2	44.0	44.8	42.4	10.1	32.6	**34.5**	**4.0**
30	33.9	49.8	30.4	40.7	50.5	40.7	13.7	60.4	**40.0**	**5.1**
40	32.0	103.1	36.4	35.2	52.8	48.0	12.6	55.2	**46.9**	**9.4**

Table B.49 Adhesion of sickle erythrocytes treated with DETA-NO to TNF-α stimulated MECs. Average data are presented graphically in Figure 6.5, Panel B.

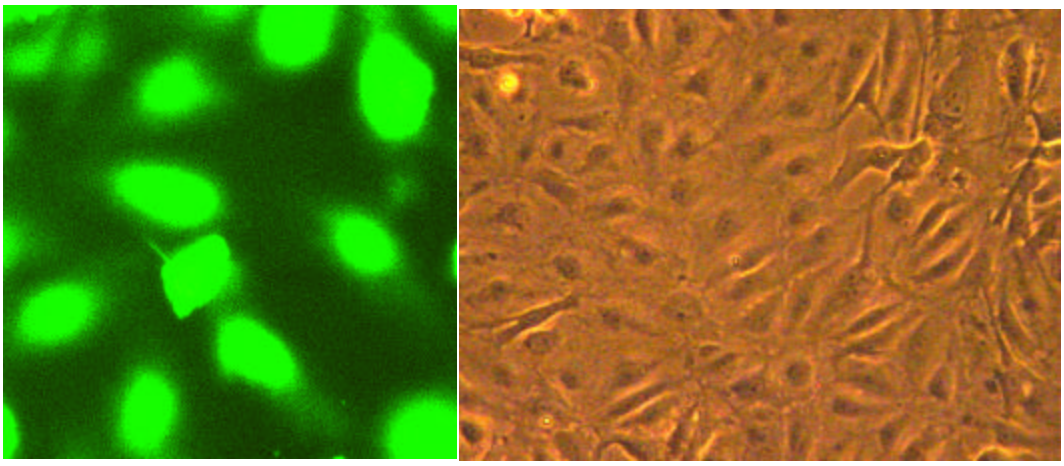
Unstimulated SSRBCs Unstimulated MECs	Experiment Number								
Perfusion Time (min)	**S49**	**S73**	**S74**	**S75**	**S76**	**S77**	**S78**	**Average**	**SEM**
1	10.1	0.0	3.7	2.1	0.0	3.2	0.0	**2.7**	**1.4**
3	5.1	9.8	16.0	8.0	8.0	5.3	10.7	**9.0**	**1.4**
5	5.5	14.2	12.0	9.1	4.4	2.4	9.8	**8.2**	**1.6**
10	8.0	29.1	35.2	12.4	14.6	10.1	14.4	**17.7**	**3.9**
20	5.3	39.0	24.0	9.8	16.0	9.6	17.5	**17.3**	**4.3**
30	2.1	35.0	43.2	9.6	12.8	8.3	18.3	**18.5**	**5.7**
40	8.0	54.4	27.4	12.4	18.8	11.6	24.8	**22.5**	**6.0**

Unstimulated SSRBCs TNF-α (6 hrs) on MECs	Experiment Number								
Perfusion Time (min)	**S49**	**S73**	**S74**	**S75**	**S76**	**S77**	**S78**	**Average**	**SEM**
1	3.4	1.5	2.3	0.8	8.8	0.9	3.5	**3.0**	**1.0**
3	18.2	2.3	16.8	5.6	17.6	3.8	13.9	**11.2**	**2.6**
5	20.8	30.7	37.7	9.0	36.7	6.0	22.9	**23.4**	**4.8**
10	32.7	29.9	74.7	25.3	34.2	16.0	25.6	**34.1**	**7.1**
20	37.8	32.0	59.1	69.3	48.8	15.3	36.6	**42.7**	**6.8**
30	41.7	21.7	31.3	82.0	75.2	27.1	41.6	**45.8**	**8.9**
40	41.7	31.2	42.3	52.0	81.0	29.9	52.4	**47.2**	**6.6**

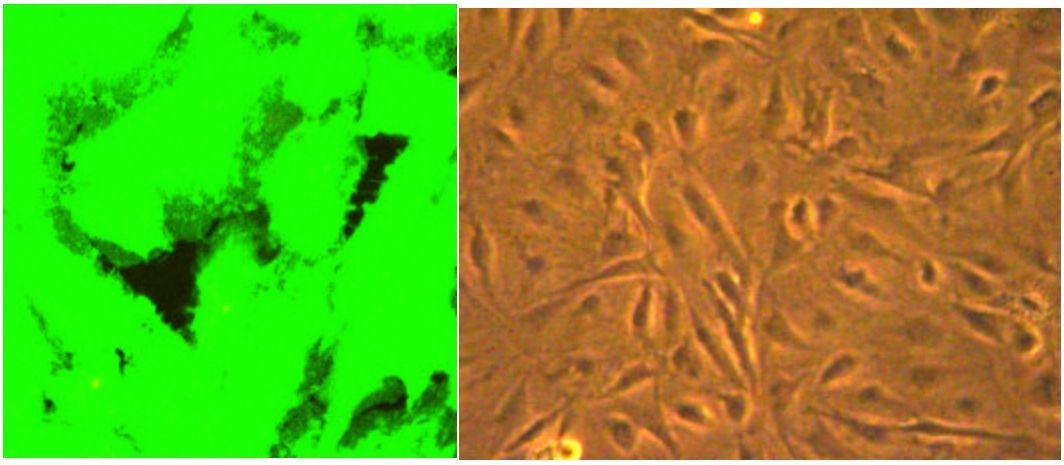
DETA-NO on SSRBCs TNF-α (6 hrs) on MECs	Experiment Number								
Perfusion Time (min)	**S49**	**S73**	**S74**	**S75**	**S76**	**S77**	**S78**	**Average**	**SEM**
1	5.6	0.0	4.0	2.0	2.7	2.5	1.5	**2.6**	**0.7**
3	4.9	4.9	9.1	16.8	8.6	2.8	3.2	**7.2**	**1.9**
5	8.0	29.1	24.5	19.4	5.8	5.3	17.4	**15.7**	**3.6**
10	10.0	33.7	28.0	9.1	46.1	15.4	19.1	**23.1**	**5.1**
20	30.6	70.1	17.8	16.0	58.7	16.7	36.9	**35.2**	**8.2**
30	22.0	25.6	28.0	26.7	87.5	20.2	18.4	**32.6**	**9.2**
40	41.7	32.8	18.4	32.9	65.3	18.2	34.5	**34.8**	**6.0**

APPENDIX C

LIGHT AND CONFOCAL MICROSCOPY PHOTOGRAPHS OF MECS TREATED WITH REAGENTS THAT AFFECT INTRACELLULAR cAMP AND NITRIC OXIDE CONCENTRATIONS

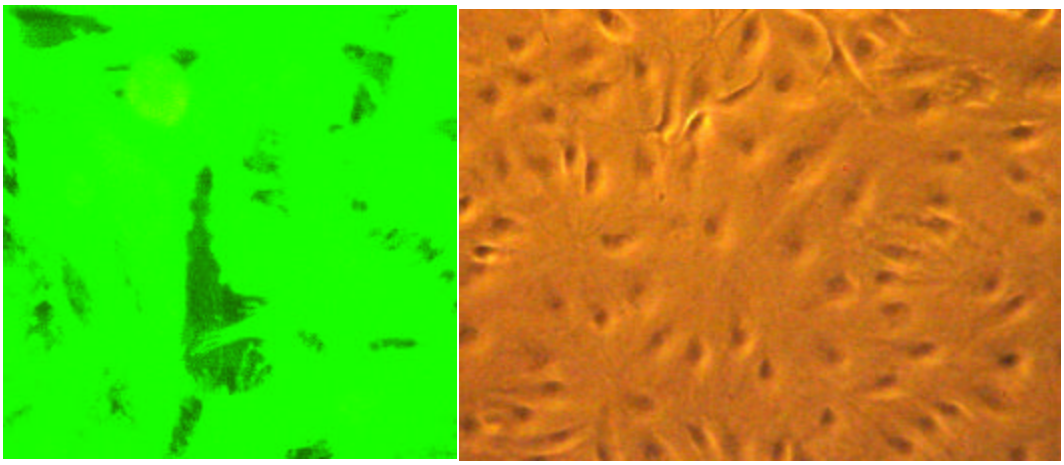


Unstimulated MECs

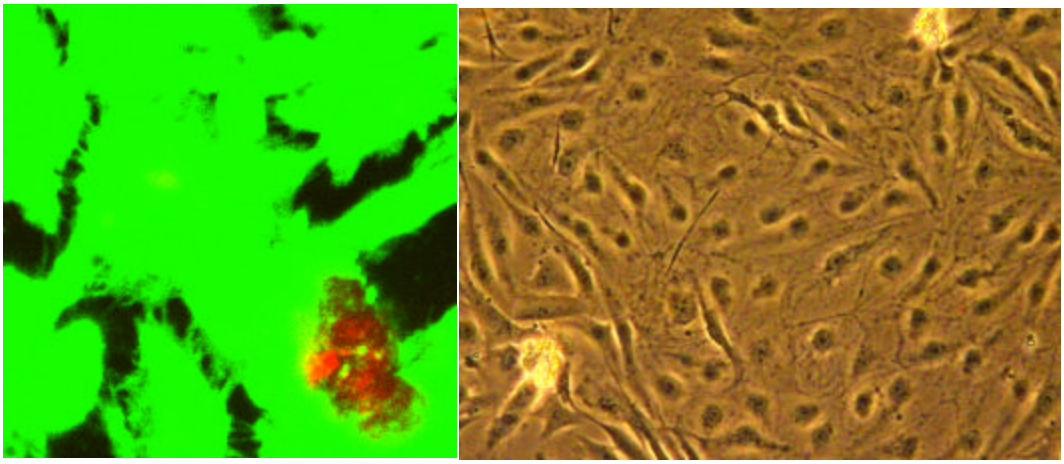


TNF-α (6 hrs)

Figure C.1 Endothelial cells after treatment with various reagents. Panels on the left show live (green) and dead cells (red) after treatment with reagents (400X). Panels on the right show light microscopy photographs after treatment with reagents (100X).

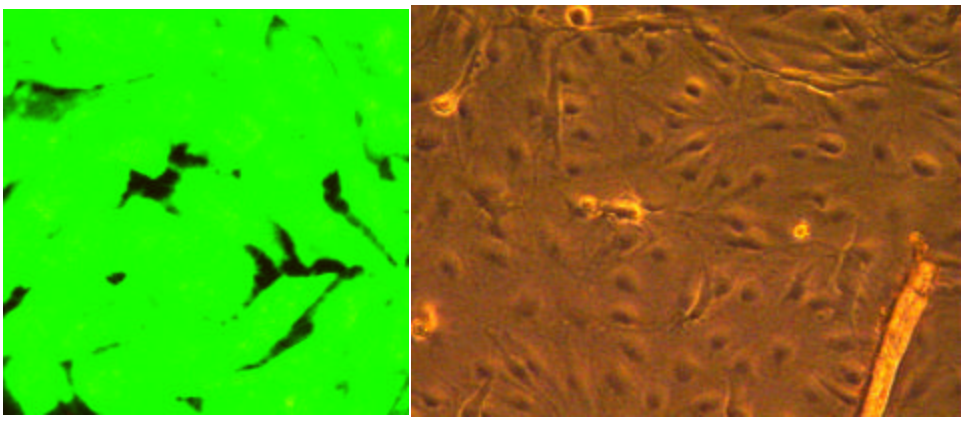


Fsk (pre + cont) + TNF-α (6 hrs)

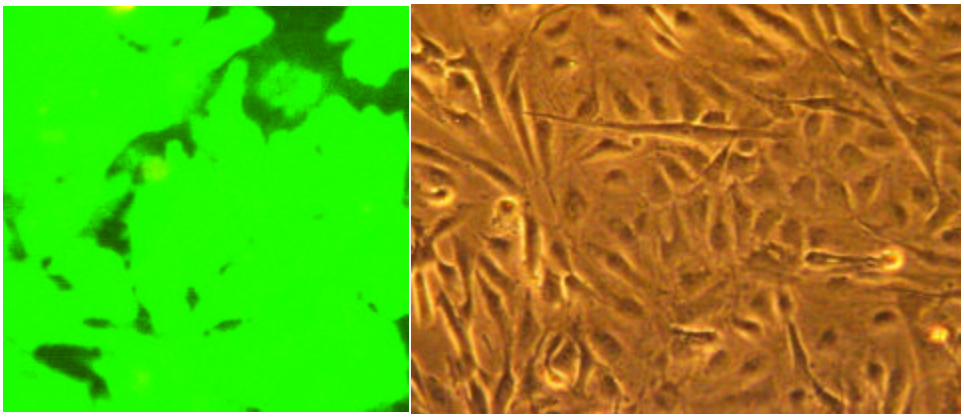


IBMX (pre + cont) + TNF-α (6 hrs)

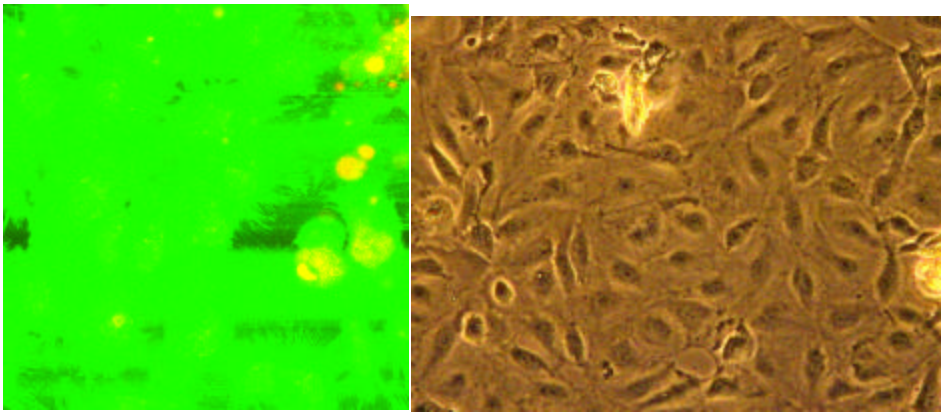
Figure C.1, continued.



Bt_2cAMP (pre + cont) + TNF-α (6 hrs)



SNP (pre + cont) + TNF-α (6 hrs)



DETA-NO (pre + cont) + TNF-α (6 hrs)

Figure C.1, continued.

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VITA

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